UNIVERSITY OF ALBERTA

Role of Carbohydrates in Enteropathogenic *Escherichia coli* Adherence

by

Rosa Pauline Vanmaele ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Medical Microbiology and Immunology

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) continue to be an important cause of diarrhea world-wide. Clinical symptoms result from bacterial colonization of the host intestinal epithelium and the formation of attaching and effacing lesions. In general, EPEC binding to epithelial cells is thought to occur by a multi-step process. My research focused on investigating host cell and bacterial factors involved in the initial attachment of EPEC to eukaryotic cells since relatively less is known about this stage of binding. Using Chinese hamster ovary (CHO) cell Lec mutants which express altered carbohydrates on their surface, I found that EPEC bound to asialo-lactosamine sequences of N-linked glycoproteins. In these experiments, I also observed that EPEC caused a characteristic detachment of CHO cell monolayers which was not due to toxicity. A possible role for sialic acid in mediating this effect was suggested by my results. Additional experiments with EPEC mutants which were defective at different steps of the attachment process revealed that early signaling events were required for CHO monolayer detachment. Further identification of specific carbohydrate sequences involved in EPEC binding was achieved by using synthetic bovine serum albumin-glycoconjugates to compete for bacterial binding to HEp-2 cells. In experiments where EPEC, strain E2348/69, was used, *N*-acetyllactosamine was the most effective inhibitor of attachment. Lewis X sequences were the next most effective inhibitor of EPEC binding, while Lewis Y structures were the least effective. In these binding inhibition experiments, the decrease in EPEC localized adherence caused by specific glycoconjugates correlated with a decrease in the expression of the bundle-forming pili structural subunit (BfpA) and intimin by the bacteria. Although preliminary, results of additional binding inhibition experiments with EPEC strains of different serotypes revealed differences in binding specificities. The reason for this

variability remains to be determined. Finally, I found that specific carbohydrates, when provided as a carbon source, also affected the expression of BfpA and intimin by EPEC, and the ability of bacteria to bind. Overall, these results suggest that carbohydrates have several roles in attachment. These roles include functioning as receptors for EPEC binding and in regulating the expression of bacterial proteins associated with adherence.

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LIST OF ABBREVIATIONS

3′-SL	3′-sialyllactose
A or Abs	absorbance
A/E or AE	attaching and effacing
AF/R1	adhesive factor/rabbit 1
BCIP	5-bromo-4-chloro-3-indolylphosphate
BFP	bundle-forming pili
Bip	bactericidal/permeability-increasing protein
BSA	bovine serum albumin
CDT	cytolethal distending toxin
ces	chaperone for *E. coli* secreted protein
cfm	class four mutant
CFU	colony forming units
CHO	Chinese hamster ovary
CMP	cytosine monophosphate
DAG	1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
eae	*E. coli* attaching and effacing
EAF	EPEC adherence factor
EDTA	ethylene diamine tetraacetic acid
ECL	enhanced chemiluminescence
EHEC	enterohemorrhagic *E. coli*

EPEC	enteropathogenic *E. coli*
esc	*E. coli* secretion
Esp	*E. coli* secreted protein
FAS	fluorescent-actin staining
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
GlcNAc-T1	*N*-acetylglucosaminyltransferase
GTP	guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
Ig	immunoglobulin
IPs	inositol phosphates
IP_3	inositol 1,4,5-trisphosphate
kDa	kilodalton
LA	localized adherence
LacNAc	*N*-acetyllactosamine
LB	Luria broth
LEE	locus of enterocyte effacement
LeX	Lewis X
LeY	Lewis Y
MBP	maltose-binding protein
MEM	minimum essential medium

mol/mol	mole/mole
NBT	nitroblue tetrazolium
ND	no data
NeuNAc	*N*-acetylneuraminic acid (sialic acid)
Omp	outer membrane protein
ORFs	open reading frames
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
per	plasmid-encoded regulator
PKC	protein kinase C
RDEC	rabbit diarrheal *E. coli*
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
sep	secretion of *E. coli* proteins
SLT	Shiga-like toxin
TBST	Tris buffered saline with Tween-20
Tir	translocated intimin receptor
TSB	tryptic soy broth
Tris	tris (hydroxymethyl) aminomethane
Tween	polyethylenesorbitan monolaurate
UDP	uridine diphosphate
vol/vol	volume/volume
wt/vol	weight/volume
XTT	3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)

Yop	*Yersinia* outer membrane protein

CHAPTER 1

General Introduction

1. INTRODUCTION

Escherichia coli are a heterogeneous group of bacteria that can be isolated from both intestinal and extraintestinal sites of humans. *E. coli* isolated from the intestinal tract can generally be grouped into two categories; those comprising the normal commensal flora, and those which are pathogenic and cause disease in the host. Normal flora *E. coli* are those which comprise a relatively fixed population of organisms regularly found in the intestine of the host. These bacteria do not usually cause disease unless they are displaced from their niche into, for example, the blood or tissues (56). These organisms are important for host health through their ability to provide their hosts with nutritional metabolic byproducts. They also discourage colonization of the intestinal mucosa by pathogenic species of bacteria and stimulate development of the host immune system (14).

Pathogenic strains of *E. coli*, on the other hand, colonize a host transiently (56). If present in sufficient numbers, their presence alone can cause disease in a host. Colonization of the intestinal tract by pathogenic *E. coli* usually results in pathology which varies in clinical presentation depending on the virulence properties of the bacteria. The enterovirulent *E. coli* are currently divided into sub-groups based on pathogenic mechanisms. These groups include enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli*. The ability of diffusely-adherent *E. coli*, an additional category, to cause disease remains to be resolved (83, 116). Since EPEC isolated from patients do not express any recognized virulence factors such as toxins or invasiveness in the Sereny test, some speculated that these bacteria might belong to another group of enterovirulent *E.*

coli which had lost their ability to express these factors (45, 74). In 1978, however, the pathogenicity of EPEC was resolved by the work of Levine *et al.* who demonstrated that EPEC caused diarrhea in adult volunteers in the absence of any known virulence factor expression (74).

1.1. CHARACTERISTICS OF INFECTION

1.1.1. Epidemiology

EPEC is a cause of diarrhea world-wide, with disease occurring most frequently in developing countries (26, 45, 116). In these countries, disease occurs regularly in hospitals, clinics, as well as in the general community. EPEC outbreaks in developed countries, on the other hand, usually consist of sporadic, isolated incidents localized to neonatal nurseries of hospitals or day-care centers. Infants less than 6 months of age are most often affected, although EPEC is also capable of causing disease in children and adults. The transmission of EPEC infections is thought to occur primarily by the fecal-oral route as a result of contact with infected individuals, or with contaminated surfaces or food. The isolation of EPEC from asymptomatic individuals, however, has led to speculation that these individuals are carriers who can also spread infection.

1.1.2. Clinical Symptoms

Clinical symptoms of EPEC infection in children consist of diarrhea which varies in duration (days to months) and severity (97, 116). In addition to profuse watery stool, symptoms include dehydration, fever, vomiting and weight loss. In protracted or severe cases, disease is often associated with the delayed growth of children, metabolic acidosis (decrease in blood pH resulting from a loss of bicarbonate (12, 41)), and in extreme cases, death. Adults participating in volunteer studies of EPEC infection displayed symptoms similar to those observed in children. However, unlike the disease in children, illness in adults usually occurs for a period of several days only.

Results of volunteer studies indicate that, at least in adults, a relatively large infectious dose of organisms is required to produce symptoms which typically occur 7 to 16 hours after infection (26, 74). For ethical reasons, similar information for EPEC infection in children is not available. It is speculated however, that a much lower number of organisms is required to infect children since transmission frequently occurs in nosocomial or day-care facilities (26). Treatment for EPEC infection usually consists of rehydration therapy and if necessary, nutritional supplementation. Antibiotics are often used to treat EPEC infection though their overall effectiveness is uncertain (116).

1.1.3. Histopathology

Biopsies from children infected with EPEC reveal that the bacteria predominantly colonize the small intestine, although the large intestine can also be involved presumably due to bacterial overgrowth (9, 45, 97, 110). The primary histopathological consequence of colonization is the atrophy of microvilli at sites of bacterial attachment and the close association of bacteria with pedestal-like structures formed by enterocytes. This characteristic effect is referred to as an "attaching and effacing" (A/E) lesion (20, 81, 97, 110). Other features include the deterioration of the terminal web (apical region beneath microvilli consisting cytoskeletal proteins which are physically associated with the central actin filaments of microvilli) of A/E lesions, a reduction in mucosal thickness, and a general disordered arrangement of enterocytes. Bacteria are rarely found within intestinal epithelial cells or in the lamina propria, suggesting that EPEC are not invasive. An infiltration of inflammatory cells into the lamina propria of the intestine is also frequently observed during EPEC infection.

1.2. IDENTIFICATION OF EPEC

Enterovirulent *E. coli* strains were traditionally identified by serology based on the O (somatic) and H (flagellar) typing method developed by Kauffmann (61). According to this method, strains currently classified as EPEC based on the possession of specific virulence factors, are of the following serotypes: O44 (H34), O55 (H-,H6,H7), O86 (H34), O111 (H-,H2,H7,H12,H21), O114 (H2), O119 (H6), O126 (H-,H2), O127 (H-,H6,H21), O128 (H2,H7,H12), O142 (H6),O158 (H23). Isolates of EPEC serotypes O18 (H7, H14), O44 (H18), O125 (H21) and O126 (H27), which were previously considered EPEC, have been shown to not possess virulence factors typical of EPEC (26).

Since the serotype of an organism does not always correlate with the possession of virulence factors, however, assays were developed to identify EPEC based on their characteristic pattern of attachment to epithelial cells (93). The first assay involves the attachment of EPEC as microcolonies to localized sites on the epithelial cell surface, in a pattern termed localized adherence (LA) (100, 101). Briefly, after incubating bacteria with tissue culture cells for 3 hours, the cells are washed, fixed and then stained. Epithelial cells with bound microcolonies consisting of 5 or more bacteria are considered positive for LA (113). The second assay, referred to as the fluorescent-actin staining (FAS) test, was developed by Knutton *et al.* (69, 70). In this assay, bacteria are incubated with tissue culture cells for 3 (or 6) hours. After washing, the cells are permeabilized and then incubated with fluorescein isothiocyanate (FITC)-phalloidin which binds to filamentous actin. The cells are then viewed with a fluorescence microscope for the accumulation of actin beneath adherent bacteria.

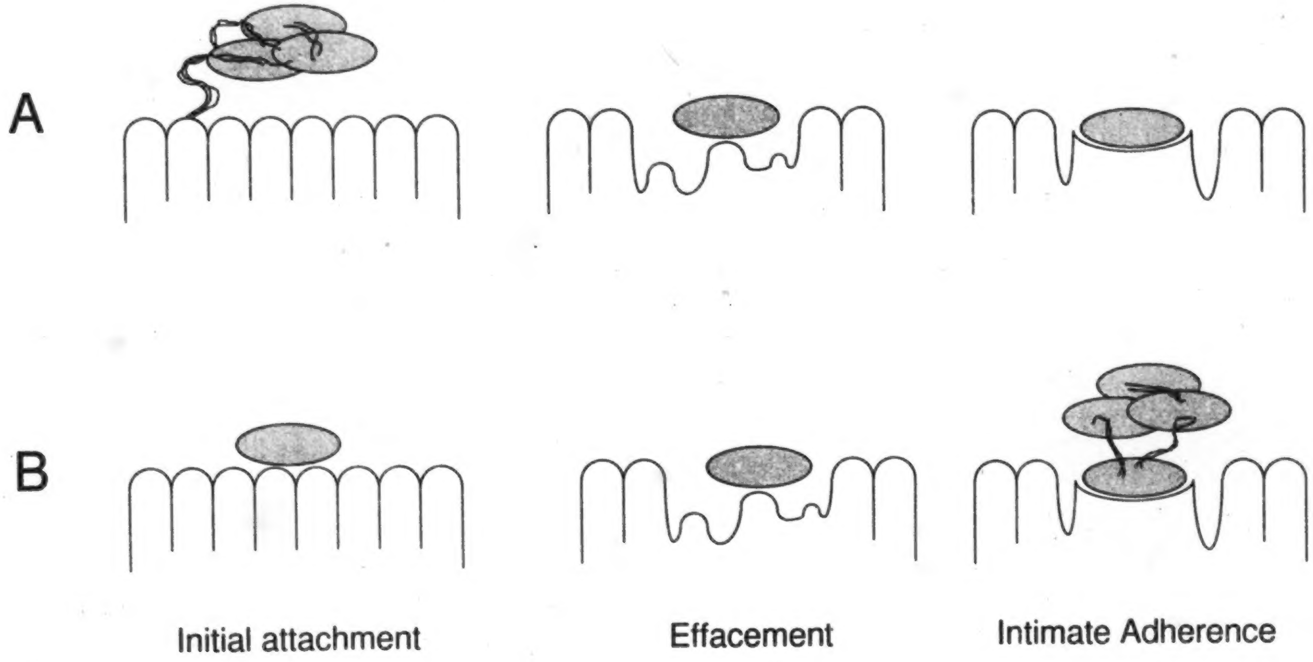
More recently, the attachment factors responsible for these phenotypes have provided the basis for DNA techniques to identify EPEC. The ability of EPEC to attach as microcolonies requires the production of bundle-forming pili (BFP), which are encoded by a large EPEC adherence factor (EAF) plasmid (6, 47). Expression of the bacterial protein intimin is required for actin rearrangement observed following EPEC binding (57, 59). DNA probes or polymerase chain reaction primers for detecting gene sequences for BFP (46, 79, 82), the EAF plasmid (42, 58, 84) or intimin have all been described (43, 59, 75).

Recently, Donnenberg defined EPEC strains as those *E. coli* which "attach intimately to and efface the microvilli of enterocytes but do not produce high levels of Shiga-like toxins (SLTs)" (26). This definition was derived from the observation that not all EPEC strains display the LA phenotype under standard assay conditions (i.e. 3 hours co-incubation of EPEC with tissue culture cells) (70). Therefore, tests to identify EPEC should now be the FAS test, which detects actin accumulation, and the Shiga-like toxin cytotoxicity assay (4, 69). Nevertheless, while the absence of BFP or EAF plasmid sequences does not exclude identification of an isolate as EPEC, the presence of these sequences is highly suggestive of EPEC (7, 46, 79, 84).

1.3. PATHOGENESIS

Clinical symptoms caused by EPEC result from bacterial attachment to the intestinal epithelium. Donnenberg and Kaper proposed a model in which EPEC attachment involves a three-step process (Figure 1.1.A) (30). The initial step consists of initial, non-intimate attachment of bacteria as microcolonies to epithelial cells, and is followed by the effacement of microvilli. In the final stage of attachment, bacteria are found in close proximity to cup-like pedestal structures formed by epithelial cells. The latter steps of effacement and intimate attachment results in the characteristic A/E lesions associated with EPEC (81). A modified version of this model has recently been suggested by Hicks *et al.* In their model, three-dimensional microcolonies of EPEC are thought to develop after intimate attachment has occurred (Figure 1.1.B) (52).

Figure 1.1. Two models of EPEC attachment to epithelial cells. The model of Donnenberg and Kaper (A) proposes that EPEC attach initially as microcolonies to epithelial cells. This leads to the effacement of microvilli and the subsequent intimate attachment of the bacteria to pedestal-like structures formed by epithelial cells. A modified version of attachment is proposed by Hicks *et al.* (B). In this model, microcolonies of bacteria are thought to develop once the bacteria have adhered intimately to epithelial cells. This figure is similar to that depicted by Hicks *et al.* (30,52).


A
B
Initial attachment
Effacement
Intimate Adherence

1.3.1. Initial Attachment

1.3.1.1. Bacteria

Cravioto *et al.* initially demonstrated that EPEC adhered to HEp-2 cells in greater numbers than other groups of *E. coli* studied, and that this adherence was not due to type I fimbriae (23). Type I pili are structures which are expressed with similar frequency by pathogenic and non-pathogenic *E. coli* strains, and whose binding is inhibited by D-mannose (60). Subsequent investigations resulted in several different structures being proposed as EPEC adhesins. These structures included unidentified non-fimbrial (102) and fimbrial adhesins (68, 114, 118), fimbriae with N-terminal sequence homology to the fimbriae of uropathogenic and diffusely-adhering *E. coli* (48), and a 32 kDa outer membrane protein (99) (later reported to be OmpF (17)). However, the observation that EPEC grown in tissue culture medium attached to epithelial cells in a LA pattern (101, 113), and that this phenotype was encoded by a large EAF plasmid (6), led to the identification of a structure required for this pattern of binding.

In 1991, Giron *et al.* described unique rope-like structures, termed BFP, (47) which appeared by scanning electron microscopy, to intercourse between bacteria to form microcolonies, and to attach the microcolonies to HEp-2 cells. Their role in attachment was supported by observations that antibodies raised against purified BFP partially inhibited EPEC attachment, and mutants lacking the EAF plasmid did not express BFP.

Characterization of the EAF plasmid revealed that two regions of the plasmid were required simultaneously to confer the LA phenotype (85). The role of one of the regions, while not well defined, contains a regulatory region termed *per* (plasmid encoded regulator), which increases the expression of EPEC virulence factors (49, 65, 108). The second region contains a fourteen gene cluster which codes for the biosynthesis of BFP (104, 108). The first gene of this *bfp* locus, *bfpA*, encodes for an 18.5 kDa protein which is the structural subunit of BFP (29, 103). The roles of proteins encoded by three other open reading frames (ORFs) have also been examined with the following results: *bfpP* encodes a prepilin peptidase which cleaves the leader sequence of the precursor pilin protein (prebundlin) to form the mature (bundlin) protein (122); *bfpB* encodes a 58 kDa lipoprotein required for the assembly of mature pili subunits into BFP filaments (91); the *bfpF* gene product reduces EPEC autoaggregation, which is proposed to facilitate bacterial dispersal in the intestine (3, 15). The roles of the gene products of the remaining ORFs of the *bfp* cluster are, at present, either speculative based on homologies to similar sequences involved in pili biogenesis of other bacteria, or are unique and remain to be determined (104, 108). In addition to genes of the *bfp* locus, a chromosomal *dsbA* (11) gene is required for the stability of the BfpA protein, presumably by promoting the formation of a disulfide bond between the two cysteine residues of the protein (121).

Until recently, BFP were thought to have a dual role in attachment; the first being to promote the formation of microcolonies, and the second to attach the bacteria to epithelial cells. This latter role was based on experimental results (i) of scanning electron micrographs in which BFP appeared to link bacteria to HEp-2 cells (47), (ii) that mutations in *bfpA* abrogated EPEC attachment (29), and (iii) that an intimin-deficient mutant of EPEC which expressed BFP adhered to HEp-2 cells (52). However, whether BFP serves as an adhesin *in vivo* requires further investigation since Hicks *et al.* recently demonstrated that EPEC mutants which expressed BFP, but were incapable of intimate attachment, did not adhere to *in vitro* organ culture of pediatric intestinal tissue (52).

1.3.1.2. Eukaryotic Cell Receptors for Initial Attachment

Studies by several research groups have implicated carbohydrate structures as host cell receptors for EPEC. In studies where soluble compounds were used to inhibit attachment, *N*-acetylgalactosamine (99), GM_3 gangliosides (53), or fucosylated tetra- and pentasaccharides (24) were found to be the most effective inhibitors of EPEC binding (Table 1.1.). These structures, excluding GM_3 gangliosides, presumably inhibited initial attachment since inhibition was measured as a decrease in the numbers of LA EPEC bound to epithelial cells. Alternatively, when glycolipids were layered onto thin layer chromatography plates, EPEC preferentially recognized the GalNAcβ(1→4)Gal portion of asialo-GM_1 and asialo-GM_2 sequences (54). These studies also suggested that the sequences were involved in initial attachment since LA-negative mutants did not bind to these sequences. In addition to these results, work performed in our laboratory suggested that EPEC recognize lactosamine sequences on eukaryotic cells. These results were obtained using two approaches. In the first method, we examined EPEC LA to Chinese hamster ovary cells or mutants of these cells which express altered oligosaccharide structures on their surface. Results of these studies suggested that asialo-lactosamine sequences on N-linked glycoproteins were sufficient for EPEC binding. As well, our results supported a minor role for O-linked glycoproteins or glycolipids in attachment (refer to Chapter 2) (112). In subsequent work in which bovine serum albumin-glycoconjugates were used to inhibit EPEC LA to HEp-2 cells, we found that *N*-acetyllactosamine and Lewis X oligosaccharide sequences were inhibitory for bacterial attachment (refer to Chapter 3).

Table 1.1. Oligosaccharide Sequences Proposed to be Involved in EPEC Binding

Oligosaccharide	[a]Inhibitory Sequence	Reference	Reference #
N-acetylgalactosamine	GalNAc	Scaletsky *et al.*, 1988	99
difucosyllactose lacto-*N*-fucopentaose	Fucα(1-2)Galβ(1-4)[Fucα (1-3)]Glc Fucα (1-2)Galβ (1-3)GlcNAcβ (1-3)Galβ (1-4)Glc Galβ (1-3)[(Fucα (1-4)]GlcNAcβ (1-3)Galβ (1-4)Glc Galβ (1-4)[(Fucα (1-3)]GlcNAcβ (1-3)Galβ (1-4)Glc	Cravioto *et al.*, 1991	24
asialo-GM_1 asialo-GM_2	[b]Galβ (1-3)GalNAcβ (1-4)Galβ (1-4)Glcβ (1-1)Cer GalNAcβ (1-4)Galβ(1-4)Glc(1-1)Cer	Jagannatha *et al.*, 1991	54
GM_3	Siaα(2-3)Galβ (1-4)Glcβ (1-1)Cer	Idota and Kawakami, 1995	53
lactosamine of N-linked glycoproteins O-linked glycoproteins or glycolipids	Galβ(1-3/4)GlcNAc not known	Vanmaele *et al.*, 1995	112
N-acetyllactosamine Lewis X	Galβ(1-4)GlcNAc Galβ (1-4)[Fucα (1-3)]GlcNAc	Vanmaele *et al.*, submitted 1998	-

[a] *N*-acetylgalactosamine=GalNAc; fucose=Fuc; galactose=Gal; *N*-acetylglucosamine=GlcNAc; glucose=Glc; Sia=sialic acid; ceramide=Cer

[b] Underlined portion of sequence proposed to be inhibitory

1.3.2. Formation of A/E Lesions

1.3.2.1. Bacteria - Effacement

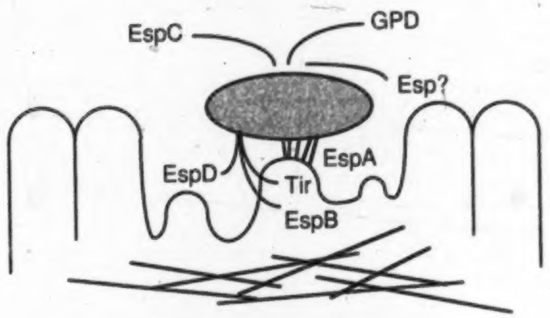
EPEC secrete several proteins which trigger signal transduction pathways in epithelial cells (Figure 1.2.A) (51, 55, 65). Second messengers generated by these signals result in the formation of A/E lesions, and are thought to contribute to the development of diarrhea (refer to section 1.3.2.3.) (8, 10, 13, 33, 40, 76). One of the proteins, EspB (E. *coli* secreted protein) (37 kDa) (formerly EaeB for E. *coli* attaching and effacing; Table 1.2.) was first identified by its role in intimate attachment (32). Subsequently, the expression of this protein was shown to be required for inducing tyrosine phosphorylation, the generation of inositol phosphates, and cytoskeletal rearrangement in epithelial cells (39). Studies revealed that following EPEC attachment, EspB becomes localized to the plasma membrane and cytoplasm of epithelial cells (117). While the precise role(s) of EspB in signaling is not known, Wolff *et al.* speculated that, based on homology to the YopB protein of *Yersinia* spp, EspB which localizes to the membranes of cells might function to create pores in epithelial cell membranes. Two additional secreted proteins, EspA (25 kDa) (66) and EspD (40 kDa) (72), are also required for signaling. Recently, EPEC were shown to produce filaments containing EspA that extended from the surface of bacteria to epithelial cells (71). Although the role of these structures in signaling is also not known, their involvement may be indirect since the expression of these structures is required for the translocation of EspB into epithelial cells (71). The role of EspD in signaling also remains to be determined. However, since an *espD* mutant secretes lower levels of EspA and EspB, it is possible that EspD is also indirectly involved in inducing intracellular signals (72).

Recently, the receptor for intimin, which was previously believed to be a host cell protein (Hp90), was also shown to be a protein secreted by EPEC (25, 63, 94, 96). This

Figure 1.2. EPEC factors and host cell processes during the formation of A/E lesions. The effacement step of attachment (A) is associated with the secretion of proteins by EPEC. Several of these proteins initiate signal transduction processes in epithelial cells, which leads to the generation of intracellular messengers and a diffuse accumulation of cytoskeletal proteins beneath the bacteria. Following the effacement of microvilli, EPEC adhere intimately to host cells through the bacterial protein intimin (B). Additional signal transduction processes are generated during this stage of attachment, which focuses the cytoskeleton to form a pedestal-like structure. Ultimately, EPEC become intimately associated with the pedestals to form A/E lesions.

A

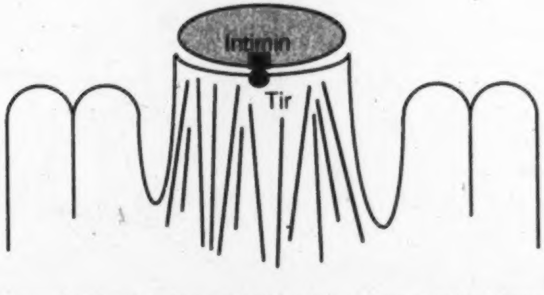
Effacement


EspC
GPD
Esp?
EspD
EspA
Tir
EspB

phosphorylation of Tir
dephosphorylation of proteins
inositol phosphates ↑
actin (other cytoskeletal proteins?) accumulation

B

Intimate Attachment


Intimin
Tir

phosphorylation of phospholipase $\gamma 1$ and other protein(s)
intracellular calcium ↑ (?)
protein kinase C activation
focused actin (and other cytoskeletal proteins)

Table 1.2. Terminology for EPEC Virulence Genes

(from Elliott *et al.*, 1998 (34))

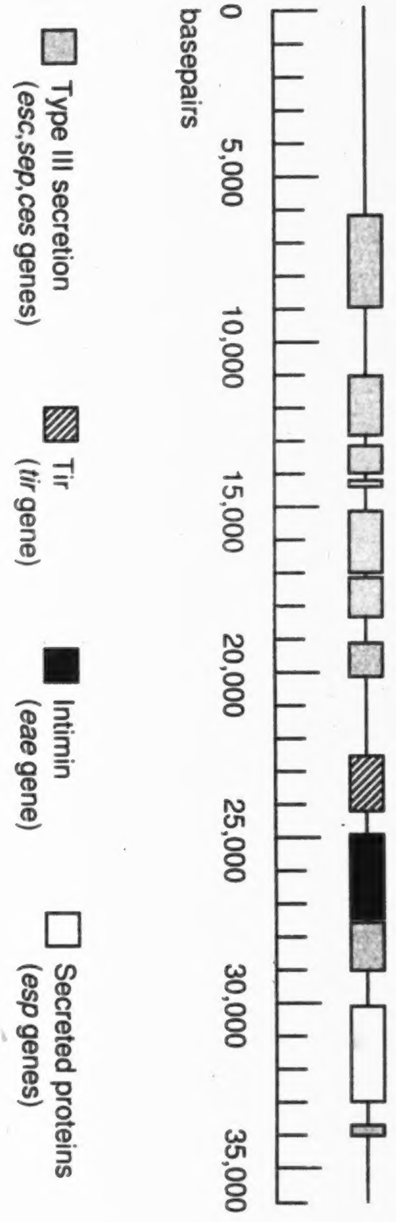
Previous Name	Current Name
eaeA	*eae*
-	*espA*
eaeB	*espB*
-	*espD*
-	*tir*
sepC	*escC*
-	*escD*
-	*escF*
sepD	*escJ*
sepB	*escN*
sepI	*escR*
sepH	*escS*
sepG	*escT*
sepF	*escU*
sepA	*escV*
sepE	*cesD*
-	*sepQ*
-	*sepZ*

protein, referred to as Tir (translocated intimin receptor) (78 kDa) (or EspE (25)), is translocated into host cells where it becomes phosphorylated at tyrosine residues and then serves as the receptor for intimin. The gene for this protein, as well as those of the other secreted proteins EspA, EspB, and EspD, are located within the LEE (locus of enterocyte effacement) region of the EPEC chromosome (Figure 1.3.) (34, 77, 78). This locus, which is referred to as a "pathogenicity island" because the genes located within this region are associated with virulence (50), contains all of the genes required for the effacement and intimate attachment steps of EPEC binding.

In contrast to the proteins just described, EPEC secrete three other proteins for which the corresponding genes are not located within the LEE region. EspC (110 kDa), which has sequence homology to IgA proteases, does not appear to have a role in adherence, signaling or invasion of host cells (65, 106). Of the two remaining proteins, one has N-terminal amino acid sequence homology with glyceraldehyde 3-phosphate dehydrogenase (39 kDa), while no homologs of the second protein (40 kDa) have been identified (65). Since these latter proteins are not encoded in the LEE region, they are thought to not be required for the formation of A/E lesions (72).

In parallel with studies demonstrating that EPEC secreted proteins which were required for virulence, Jarvis *et al.* identified four ORFs, also within the LEE region (Figure 1.3.), which were homologous to genes of type III secretory pathways of other enteric pathogens including *Salmonella*, *Shigella*, and *Yersinia* species (37, 55, 73). For these enteric pathogens, this system is used to secrete virulence-associated proteins which exert their overall effect(s) by acting on host cell factors. At present, approximately 20 genes are thought to comprise the secretory machinery of this pathway. Proteins secreted by this pathway typically: (i) are *sec*-independent (86); (ii) have an amino-terminal leader sequence which targets the protein to the secretion machinery, but is not cleaved from the

Figure 1.3. The general structure of the LEE region of the EPEC chromosome (34). Functional regions which have been identified to date are indicated. These regions are: (i) the genes coding for a type III secretory pathway responsible for the secretion of virulence factors; (ii) the gene encoding intimin, which is an EPEC outer membrane protein that focuses host cell cytoskeletal proteins beneath adherent organisms and is an adhesin; (iii) the genes for the secreted proteins which initiate signal transduction processes in host cells. Tir is a secreted protein which is translocated into host cells and becomes the receptor for intimin.


0
5,000
10,000
15,000
20,000
25,000
30,000
35,000
basepairs
Type III secretion (*esc,sep,ces* genes)
Tir (*tir* gene)
Intimin (*eae* gene)
Secreted proteins (*esp* genes)

secreted protein; (iii) require chaperones. A role for this pathway in EPEC virulence was supported by the observation that a mutation in the *escN* (*E. coli* secretion) (formerly *sepB* for secretion of *E. coli* proteins; Table 1.2.) gene of this pathway abrogated secretion of all but one of the secreted proteins. EPEC proteins requiring this type III system for their export include EspA, EspB, EspD and Tir (25, 63, 65, 66, 72). To date, 12 genes have been identified in EPEC which are speculated to be involved in protein secretion (34). Of these, the *cesD* (chaperone for *E. coli* secreted protein (34)) (formerly *sepE;* Table 1.2.) gene product, is thought to be a chaperone protein required for the secretion of EspB and EspD (115).

1.3.2.2. Bacteria - Intimate Attachment

Following the effacement of microvilli, the formation of actin pedestals characteristic of A/E lesions requires the bacterial protein intimin (Figure 1.2.B) (57, 59, 94). Intimin is a 94 kDa outer membrane protein encoded by the *eae* (formerly *eaeA;* Table 1.2.) gene of the LEE locus (Figure 1.3.). Expression of this protein is necessary to focus host cytoskeletal proteins which accumulate beneath the organisms into pedestals, and for bacteria to become intimately associated (less than 10 nm separation) with this structure (81). Based on serological and genetic techniques, EPEC intimins have been classified into three groups (1, 2, 57). Despite these variations in antigenicity and gene sequences however, the proteins are believed to be functionally equivalent. The greatest diversity among these proteins occurs within the C-terminus, which is also proposed to be the host cell binding domain (43).

Recently, Knutton *et al.* demonstrated that intimin expression is down-regulated following the formation of A/E lesions (67). One possible interpretation of these results is that, once bacteria have achieved their goal of becoming intimately associated with host cells, continued expression of this protein is no longer required in order to remain attached. Alternatively, since intimin is immunogenic (18), this may be a mechanism by which EPEC are able to evade the host immune response.

1.3.2.3. Host Cells in A/E Lesion Formation

Early studies by Knutton *et al.* demonstrated that actin accumulated beneath EPEC at the sites of A/E lesions (69). Subsequent studies by other laboratories revealed that the actin-associated proteins α-actinin, talin, ezrin (38) and myosin light chain (13) also accumulate beneath adherent bacteria. While signaling processes prior to intimate attachment result in the accumulation of cytoskeletal protein(s) at sites of bacterial attachment, the expression of intimin is required to focus these components into a pedestal-like structure (Figures 1.2.A and B) (94). Thus far, two eukaryotic cell receptors have been described for intimin. One is the recently described Tir protein which is translocated into host cell membranes (25, 63, 96), while the second is the β_1 integrins of host cells (44).

In addition to cytoskeletal rearrangement, EPEC have been reported to cause increases in intracellular calcium and inositol phosphates (IPs) in host cells (10, 33, 40), and to alter the phosphorylation of several host cell proteins (8, 13, 64, 76, 94). Overall, signaling appears to involve a two stage process (Figure 1.2.A and B), with the latter stage requiring intimate attachment to proceed (64). Based on what has been elucidated thus far, early signaling events involve the tyrosine-phosphorylation of Tir, which appears to be necessary for the generation of other intracellular signals (40, 96). This then leads to the release of IPs (40) and the dephosphorylation of several host cell proteins (64). Following intimate attachment, increases in intracellular calcium (33), activation of protein kinase C

(PKC) (22), and tyrosine-phosphorylation of phospholipase C-γ1 (64) and other, as of yet, unidentified protein(s), occur. A model for the generation of signals following intimate attachment proposes that EPEC binding activates phospholipase C-γ1 which then cleaves phoshatidylinositol 4,5-bisphosphate to form inositol 1,4,5-tris-phosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 stimulates the release of calcium from intracellular stores which, in conjunction with DAG, activates host cell protein kinase(s) to phosphorylate cellular proteins (9, 22). Whether similar processes are associated with the early (i.e., prior to intimate attachment) production of IPs remains to be determined. Of note, Bain *et al.* recently proposed that increases in intracellular calcium observed after EPEC bind to epithelial cells is a result of bacterial toxicity to eukaryotic cells, and not a signal involved in cytoskeletal reorganization (5).

Despite the identification of eukaryotic intracellular signals generated by EPEC, their role in pathogenesis has not been confirmed. However, it is thought that increases in intracellular calcium levels activates calcium-dependent actin-severing proteins which leads to the depolymerization of actin. This, in turn, results in the effacement of microvilli (10). As well, calcium, in conjunction with DAG, is believed to activate PKC which then phosphorylates proteins involved in ion transport (10, 22, 33). This then might be a mechanism by which ion secretion by epithelial cells is altered and diarrhea results. Protein phosphorylation by PKC or myosin-light chain kinase might also contribute to diarrhea by altering the permeability of tight junctions (22, 76, 119).

1.3.3. Invasion

Several reports demonstrated that EPEC are able to invade epithelial cells by a process involving host cell microtubules and microfilaments (27, 28, 80). This led to the suggestion that invasion might represent a "fourth step" of the three-stage model proposed by Donnenberg and Kaper (35,30). However, EPEC invasion is currently not thought to play a significant role in pathogenesis since bacteria are only occasionally, if at all, observed within enterocytes from biopsies of infected individuals (81, 97). Also, when compared to the invasive ability of enteroinvasive *E. coli*, relatively few EPEC which bind to epithelial cells actually are internalized (80, 92).

1.4. REGULATION OF EPEC VIRULENCE FACTORS

Investigations into the regulation of EPEC virulence factor expression were initiated by the observation of Vuopio-Varkila and Schoolnik that the LA phenotype of EPEC was promoted by growing the bacteria in a defined medium (113). Since then, both environmental and genetic factors that regulate the expression of EPEC virulence factors have been described.

1.4.1. Environmental Factors

In the report of Vuopio-Varkila and Schoolnik, which described the positive effect of tissue culture growth medium on EPEC attachment, the increase in attachment correlated with higher levels of BFP expression (113). Subsequently, several reports identified growth conditions that are optimal for the expression of EPEC virulence factors. Specific media components which were found to affect the expression of BFP or Esps include: calcium (BfpA and Esps), ammonium (BfpA), and $Fe(NO_3)_3$ (Esps) (62, 89). As well, results from our laboratory indicated that EPEC binding to HEp-2 cells, and the expression of BfpA and intimin, were affected by carbon source (111). Regarding general environmental conditions, the secretion of Esps was dependent on the osmolarity and pH of the tissue culture media (51, 62). Growth at 37°C was also optimal for the expression of BfpA and Esps, and for the formation of A/E lesions (62, 89, 95). Since, in several instances, the expression of different EPEC virulence factors was affected by the same environmental condition(s), these results suggest that these factors may be coordinately regulated (62).

1.4.2. Regulation of Gene Expression

An early report by Jarvis and Kaper demonstrated that the EAF plasmid enhanced intimin expression by EPEC (57). In later studies, a region of this plasmid referred to as *per*, was isolated which was responsible for this effect (49). Of the four ORFs identified, *perA* shares homology with the AraC family of regulators which have been shown to regulate the expression of virulence factors in other enteric pathogens (49, 109). In addition to up-regulating intimin expression, this locus also increased the secretion of Esps by EPEC (65). More recently, the same ORFs, only referred to as *bfpT*, *bfpV* and *bfpW* (corresponding to *perA*, *perB* and *perC*, respectively), were shown to be necessary for enhanced BfpA expression by EPEC (109).

A second mechanism by which EPEC regulates the expression of virulence factors has recently been described by Farris *et al.* (36). In their studies, they identified a GTPase, BipA (for bactericidal/permeability-increasing protein A (90)), which was required for actin rearrangement in epithelial cells. This protein also regulated flagellar motility and resistance to host defense peptides. Importantly, extracts of EPEC, but not *E. coli* K-12, stimulated tyrosine phosphorylation of BipA. Since the extent of phosphorylation of BipA modulated the nucleotide-hydrolyzing activity of this protein, these results suggested that the phosphorylation of bacterial proteins is also a mechanism by which EPEC virulence is regulated (120).

1.5. MECHANISM(S) OF INDUCING DIARRHEA

The elucidation of the specific interaction(s) between EPEC and eukaryotic cells has led to the identification of several mechanisms by which EPEC are thought to cause diarrhea. The earliest proposal was based on the histopathology of A/E lesions. In a report which characterized these lesions, Moon *et al.* suggested that the loss of microvilli associated with these lesions was likely to result in the malabsorption of ions and nutrients from the intestinal lumen (81). Presumably, this would cause water to be drawn into the lumen by osmosis and this would ultimately manifest itself as diarrhea (88).

More recently, the work of several groups suggest that EPEC also causes diarrhea by altering electrolyte balances across intestinal monolayers. Several ways in which this overall effect might be achieved have been described. One of these mechanisms, which is suggested by clinical observations that the onset of symptoms can occur within 3 hours of infection (31), involves the active secretion of electrolytes into the intestinal lumen. In support of this hypothesis, Stein *et al.* reported that EPEC caused a decrease in the resting membrane potential of epithelial cells (107), although the nature of the ions mediating this effect was not determined. Similarly, Collington *et al.* observed increases in short circuit current after EPEC bound to epithelial cells (21). Part of this response was attributed to chloride ion secretion by the cells. Although the intracellular messengers in infected cells responsible for these responses have not yet been identified, increases in intracellular calcium, and the activation of protein kinases, are proposed to be involved (8, 10, 13, 22, 33, 76, 119).

A second method by which EPEC are thought to effect electrochemical gradients is by altering the permeability of the intestinal epithelium. Consistent with this hypothesis, different research groups have shown that EPEC cause a decrease in the transepithelial resistance of polarized intestinal monolayers. This effect was attributed to an increase in transcellular (16) or paracellular permeability (87, 105), depending on the tissue culture cell line used. With respect to increased transcellular permeability, channels for the secretion of chloride ions did not appear to be involved. Increased paracellular permeability, on the other hand, was presumed to result from the increased permeability of tight junctions. Manjarrez-Hernandez *et al.* previously demonstrated that EPEC induced the phosphorylation of myosin light chain, which caused it to associate with cytoskeletal proteins of epithelial cells (76). Significantly, this phosphorylation correlated with a disruption of intestinal barrier function (119). Based on these results, it was speculated that the phosphorylation of myosin light chain causes it to interact with cytoskeletal proteins connected to tight junctions, and that a contraction of this structure results in the increased permeability of tight junctions.

Finally, the recruitment of host immune cells to sites of EPEC infection is also likely to contribute to clinical symptoms. Previous histopathological examination of EPEC infected biopsies revealed an infiltration of inflammatory cells into the lamina propia of the intestinal tissue. More recently, Savkovic *et al.* demonstrated that EPEC attachment to polarized epithelial cells caused a trans-migration of polymorphonuclear cells from the basolateral side to the apical side of polarized intestinal monolayers (98). It was speculated that an increase in paracellular permeability associated with EPEC infection might facilitate the movement of neutrophils through the monolayers, as well as the diffusion of IL-8 to the basolateral side of the monolayers to attract immune cells (119). The migration of immune cells to sites of EPEC infection is likely to contribute to diarrhea by the production of soluble mediators, especially prostaglandins, which stimulate ion secretion by the epithelial cells (19).

1.6. RATIONALE FOR EXPERIMENTS

Investigations of EPEC pathogenesis revealed that bacterial attachment is important for virulence. Previous results by several research groups suggested that EPEC recognize carbohydrate structures on the surface of epithelial cells, however the specific oligosaccharide sequences proposed to be the bacterial receptor were varied (Table 1.1.). In consideration of these earlier observations, the goals of my research were three-fold: to identify a specific oligosaccharide sequence(s) recognized by EPEC, to determine the nature of the receptor on epithelial cell surfaces, and to assess the feasibility of using these structures as a potential therapy for EPEC infection. Also, although not one of the original aims of this research, insight into the role of carbon source in EPEC attachment was also investigated.

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CHAPTER 2

Effect of Enteropathogenic *Escherichia coli* on the Adherent Properties of Chinese Hamster Ovary Cells

2. PREFACE

The discrepancies in the literature regarding carbohydrate sequences recognized by enteropathogenic *Escherichia coli* (EPEC) appeared, at least in part, to be a consequence of the different methodologies used in the experiments. In these studies, two general approaches were used to investigate EPEC binding. The first involved examining EPEC binding to glycolipids layered onto thin layer chromatography plates or enzyme-linked immuno-sorbant assay plate wells (3), while the second involved using soluble inhibitors to compete for bacterial binding to epithelial cell monolayers (1, 2, 5). Both approaches were limited with respect to their representation of interactions between bacteria and epithelial cells. The first approach was limited in that purified compounds do not reflect the complexity of an epithelial cell surface (6). With the latter approach, the inhibitors used were monovalent which are usually not representative of the multivalent interactions which are thought to be involved in microbial binding (4, 8).

Given the limitations of the previous methods, I decided to adopt an alternative model system to investigate EPEC binding. This model system employed Chinese hamster ovary cells and their Lec mutants which express altered oligosaccharide structures on their surface (7). This model has been used previously to identify carbohydrate structures bound by microorganisms. The advantages of using this system compared to those used previously were that the modified surface structures are presented within the normal architecture of the epithelial cell surface so that differences in binding to these structures indicate sequences involved in attachment. The results of these experiments, however, were interpreted with caution since glycosylation can potentially affect the expression or stability of certain surface receptors (7).

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2.1. INTRODUCTION*

Escherichia coli comprise a heterogeneous group of microorganisms with wide-ranging potential for interacting with their hosts. There are three broad categories into which the *E. coli* can be subdivided: (i) nonpathogenic *E. coli* which constitute the normal flora of the host, (ii) those organisms, like the uropathogenic *E. coli*, which are opportunistic pathogens, and (iii) those organisms which are true pathogens. The diarrheagenic *E. coli* are true pathogens and can be further subdivided into at least five, and perhaps six, groups based on defined clinical symptoms and virulence mechanisms. These groups include enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, enteroaggregative *E. coli*, and enteropathogenic *E. coli* (EPEC). Recent studies suggest that a sixth group, diffusely-adherent *E. coli*, may also be included in this list (11).

Production of clinical symptoms by EPEC is a complex, multistep process initiated by bacterial attachment to intestinal epithelial cells and involves plasmid and chromosomal gene products (11, 12). The EAF (EPEC adherence factor) plasmid codes for the ability of EPEC to attach to HEp-2 cells (2) in a typical localized adherence (LA) pattern (8, 38). Knutton *et al.* demonstrated that the EAF plasmid was involved in the initial, nonintimate adherence of EPEC to epithelial cells (26). More recently, the EAF plasmid was found to code for bundle-forming pili which cause aggregation of EPEC grown in tissue culture medium and are responsible for the LA phenotype (17, 18, 40).

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* This chapter was published in INFECTION AND IMMUNITY, Volume 63, Number 1, pages 191-198 (January, 1995) by Rosa P. Vanmaele, Margaret C. Finlayson and Glen D. Armstrong. The figure in Appendix A of this chapter is indicated as "data not shown" in the published manuscript. The results presented are the basis for a patent application "Methods for Detection and Inhibition of Enteropathogenic *Escherichia coli*". Claims related to the detection of EPEC were allowed January, 1998 (Patent No. 5858698). The remaining claims continue to be prosecuted under U.S. Patent Application Serial No. 09/059,405.

EPEC also possess chromosomal *eae* (EPEC attaching and effacing) genes (11, 22, 24, 35) which, following the initial EAF-mediated attachment of the bacteria to epithelial cells, cause attaching and effacing lesions *in vivo* (32) and *in vitro* (16, 28). These lesions are characterized by intimate association of the bacteria with the epithelial cell surface, resulting in localized destruction of the microvilli and accumulation of actin (27) and other cytoskeletal components (15, 30) at the sites of bacterial attachment. The presence of this accumulated actin forms the basis of the fluorescent actin stain (FAS) test which is used to identify EPEC (27). Jerse and Kaper demonstrated that the formation of attaching and effacing lesions is regulated in *trans* by EAF plasmid gene(s) (22).

Although HEp-2 or HeLa cells are commonly used to study EPEC LA and invasion of eukaryotic cells, we have found that Chinese hamster ovary (CHO) cells represent an alternative *in vitro* model system for studying these virulence mechanisms. Moreover, in this article, we show that EPEC cause a highly reproducible and distinctive loss of CHO cell monolayers in addition to attachment and invasion. CHO cell (Lec) mutants expressing altered surface oligosaccharide sequences (9, 10, 39) also appear to be useful for investigating the requirement for sialyl and asialo-glycans by EPEC for LA, invasion, and detachment of CHO cell monolayers.

2.2. MATERIALS AND METHODS

2.2.1. Bacteria. The relevant characteristics of all bacteria used in this study are listed in Table 2.1. Enterohemorrhagic *E. coli* O157:H7 was provided by L. Linarez (Provincial Laboratory of Northern Alberta, Edmonton, Canada). *E. coli* O26:H11 (strain H19) was

Table 2.1. Characteristics of *E. coli* Strains

E. coli Strain	Enterohem-olysin[a]	SLT[b]	Rapid Loss of CHO Cell Monolayers	FAS test		*eae* gene[c]	EAF plasmid[d]
				CHO	HEp-2		
O111:H2	-	-	+	+	+	+	+
O119:H6	-	-	+	+	+	+	+
O125:H4	-	-	-	-	-	-	ND[e]
O126:H27	-	-	-	-	-	-	ND
O142:H6	-	-	+	+	+	+	+
O157:H7	+	SLT-II/SLT-I	-	ND	ND	+	-
O26:H11	+	SLT-I	-	+	ND	+	-

[a] Determined by the method of Beutin *et al.* (5).

[b] Determined by the method of Armstrong *et al.* (1). Shiga-like toxins (SLT); 50% cytotoxic dose = 0.4 pg/ml.

[c] Determined by the method of Louie *et al.* (29).

[d] Determined by the method of Mass and Jerse *et al.* (23, 31).

[e] ND, no data.

obtained from S.M. Scotland (Division of Enteric Pathogens, Central Public Health Laboratory, London, United Kingdom). All bacteria were grown aerobically at 37°C in tryptic soy broth (TSB) (Difco, Detroit, Mich.).

2.2.2. Tissue culture. CHO-K1 (CCL 61) cells and CHO cell mutants Lec1 (CRL 1735), Lec2 (CRL 1736), and Lec8 (CRL 1737) were obtained from the American Type Culture Collection (Rockville, Md.). The phenotype of the Lec mutants was confirmed by a previously published procedure (20). HEp-2 cells were provided by L. Chui (Provincial Laboratory of Northern Alberta). All tissue culture cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% air in medium supplemented with 10% fetal bovine serum (FBS). All reagents for tissue culture were obtained from Gibco (Burlington, Ontario, Canada). HEp-2 cells were grown in minimal essential medium with Earle's salts (MEM). Unless indicated otherwise, CHO cells were grown in F-12 nutrient mixture (Ham), and Lec1, Lec2, and Lec8 cells were grown in MEM (α medium) as recommended by the American Type Culture Collection. For some experiments, however, Lec2 cells were cultured separately in F-12 nutrient mixture (Ham) for a minimum of 10 passages prior to use. Confluent monolayers were disrupted using a solution consisting of 0.25% trypsin (vol/vol) in FC buffer (0.14 M NaCl, 5.0 mM KCl, 20.0 mM Tris HCl, 5.0 mM Tris Base, 0.5 mM EDTA [pH 7.2]). The cells were resuspended in the appropriate medium, and 1.25 x 10^5 cells were added to each well of the 24-well tissue culture plates. The plates were incubated at 37°C for 24 to 72 h, depending on the experiment and cell line. Subconfluent monolayers were used for the FAS test and LA assay, while confluent monolayers were used for all other experiments.

2.2.3. Bacterial replication in tissue culture medium. Approximately 2.0 x 10^6 (5-15 μl) TSB-grown, log-phase organisms (determined by measuring culture turbidity at 600 nm) were added to 0.5 ml of FBS-supplemented F-12 nutrient mixture (Ham) per well in 24-well tissue culture plates in the absence of CHO cells. The number of viable organisms added was confirmed by performing plate counts from serial dilutions of the inocula. After incubation for 3 h at 37°C in a CO_2 incubator, aliquots were removed from the wells and serially diluted in order to determine the number of viable organisms. Individual experiments were performed in duplicate and repeated twice.

2.2.4. EPEC-induced detachment of CHO cells. Tissue culture plates were prepared as described previously using CHO, Lec1, Lec2, and Lec8 cells. After the monolayers became confluent, the tissue culture medium was replaced with 0.5 ml of fresh medium containing 10% FBS and 0.5% D-mannose (8, 27) and approximately 2.0 x 10^6 TSB-grown, log phase organisms were added to each well. Experiments were performed in the presence of D-mannose to prevent type 1 pilus-mediated bacterial attachment. Following incubation at 37°C, the monolayers were washed 3 times with phosphate-buffered saline (PBS) to remove nonadherent CHO cells. CHO cells remaining in each well were fixed for 10 min with methanol and then stained with Giemsa stain for 30 min. The monolayers were then washed three times with water to remove excess Giemsa stain, and the stained cells were lysed with 2% sodium dodecyl sulfate (SDS). Solutions of lysed cells were transferred to 96-well microtiter plates, and the absorbance of the contents from each well was recorded using a Titertek Multiskan MC microtiter plate reader set at 620 nm. The percent of monolayer remaining was calculated as follows: (A_{620} Inoculated well)/(A_{620} Uninoculated well) x 100. At least four independent experiments were performed for each strain.

2.2.5. FAS test. The FAS test (27) was done as follows for all bacteria except *E. coli* O26:H11 and O157:H7. Briefly, subconfluent HEp-2 cell monolayers were grown on 12-mm-diameter glass coverslips in 24-well plates as described above. Five µl of an overnight bacterial broth culture was then added to each well in the presence of HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered (pH 7.2) MEM with 2% FBS and 0.5% D-mannose. After incubation at 37°C in a CO_2 incubator for 3 h, the coverslips were washed 3 times with PBS. The cells were then fixed for 20 min in 3% formaldehyde and then washed another three times with PBS. Next, the formaldehyde-fixed HEp-2 cells were treated with 0.1% Triton X-100 in PBS for 5 min, and the permeabilized monolayers were washed another three times with PBS and then treated with a 5-µg/ml solution of fluorescein isothiocyanate-conjugated phalloidin (phalloidin-FITC, Sigma Chemical Company, St. Louis, Mo.) in PBS for 20 min to stain actin. After being washed three times with PBS to remove excess phalloidin-FITC, the coverslips were mounted in 80% glycerol-PBS and viewed using a fluorescence microscope fitted with a 340- to 380-nm excitation filter.

When performing the FAS test on EPEC with CHO, Lec1, Lec2, or Lec8 cells, it was necessary to reduce the cocultivation time from 3 to 2 h prior to staining the monolayers with phalloidin-FITC. Essentially the same procedure was followed for *E. coli* O26:H11 and O157:H7 except that the bacteria were cocultivated with CHO cells for a total of 6 h. At 3 h of incubation with O26:H11 and O157:H7, the CHO cell monolayers were washed and fresh medium was added to prevent bacterial overgrowth. F-12 nutrient mixture (Ham) or MEM (α medium) supplemented with 10% FBS and 0.5% D-mannose was used for cocultivation of EPEC or of O26:H11 or O157:H7 with CHO and Lec cells respectively. The monolayers were then stained with phalloidin-FITC and observed with a fluorescence microscope as described above.

2.2.6. LA assay. Subconfluent monolayers of CHO, Lec1, Lec2, and Lec8 cells were prepared on 12-mm-diameter glass coverslips in 24-well plates as described previously. Following the addition of fresh growth medium containing 10% FBS and 0.5% mannose, approximately 1 x 10^9 bacteria were added to each well and the plates were incubated for 1.5 h at 37°C. The monolayers were washed five times with PBS to remove nonadherent bacteria and then fixed and stained with Giemsa stain as described earlier. Excess stain was removed by washing the coverslips three times with water. The coverslips were air dried and mounted on glass slides for viewing using the 100x oil immersion objective of a Reichert light microscope. Between 100 to 125 randomly chosen tissue culture cells were observed and only those having five or more bacteria attached as a cluster were scored as positive for LA (40). Each experiment was repeated three times.

2.2.7. EPEC binding and invasion assays. Invasion assays were performed using confluent monolayers of CHO and Lec2 cells which were prepared as described earlier. The tissue culture medium was replaced with 0.5 ml fresh medium containing 10% FBS and 0.5% D-mannose, and approximately 2.0 x 10^6 TSB-grown, log-phase bacteria were added to each well. Following incubation at 37°C for 1.5 h, the monolayers were washed three times with PBS and then incubated with gentamycin (100 μg/ml in tissue culture medium) for 1 h at 37°C in order to kill extracellular bacteria (14). After three washes with PBS, the tissue culture cells were lysed by treating them with 200 μl of 1%

(vol/vol) Triton X-100 in PBS for 5 min at 37°C. The lysed suspensions were then diluted to 1 ml with TSB, and the number of intracellular bacteria was determined from serial dilutions of this mixture.

For bacterial binding assays, the above procedure was followed except that incubation of the monolayers with gentamycin followed by three washes with PBS was omitted. Both assays were repeated four or five times.

2.2.8. Statistical analysis. Statistical analyses (nonparametric Wilcoxon test or *t* test) were performed using SYSTAT software (Evanston, Ill.) on a Macintosh Plus computer.

2.3. RESULTS

2.3.1. Detachment of CHO and Lec cell monolayers. Of all the bacteria tested in this assay, only the EPEC isolates (O111:H2, O119:H6, and O142:H6) caused detachment of CHO cell monolayers within 2 to 4 h of cocultivation (Figure 2.1.). The large error bars in this figure were the result of interexperimental variation in the time (± 30 min) of onset of the effect (Figure 2.2.). However, regardless of the time of onset of monolayer detachment, the rate at which detachment occurred was highly reproducible and always greater than that observed in CHO cells infected with non-EPEC.

E. coli O125:H4 and O157:H7 caused essentially no detachment of CHO cell monolayers over the 6-h incubation period, whereas *E. coli* O26:H11 and O126:H27 caused gradual detachment of the monolayers after 3 to 4 h of incubation. However, the changes in the adherence of CHO cells caused by *E. coli* O126:H27 and O26:H11 were not characteristic of the rapid changes caused by EPEC.

Lec1 CHO cell mutants lack an *N*-acetylglucosaminyltransferase (GlcNAc-T1)

Figure 2.1. Effect of bacteria on adherent properties of wild-type CHO cells. After incubating with bacteria for various times, CHO cells remaining attached to the tissue culture wells were fixed and stained with Giemsa stain. Stained cells were then lysed with 2% SDS, and the A_{620}s of these solutions were used to calculate the percent monolayer remaining relative to uninfected monolayers.

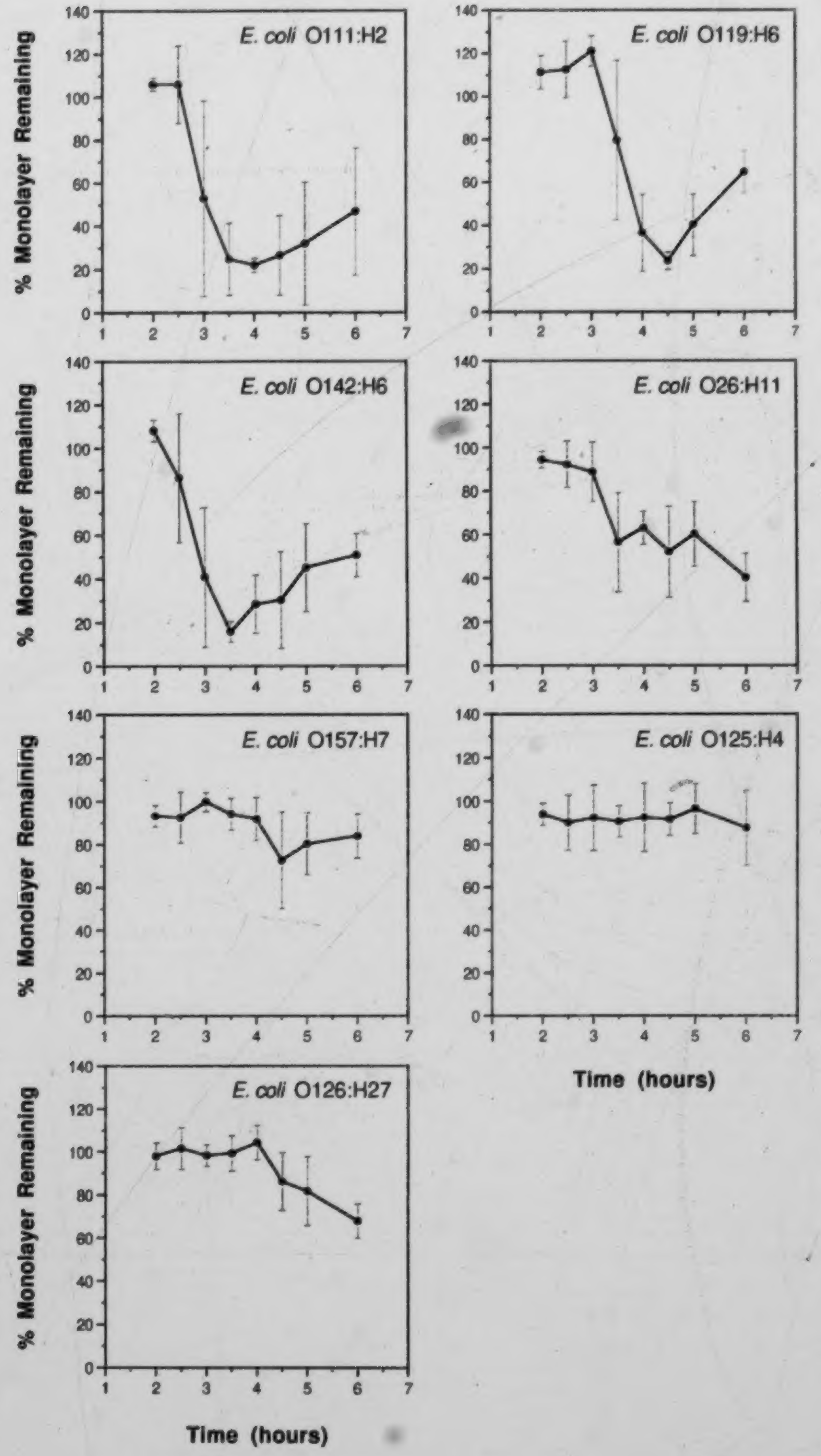
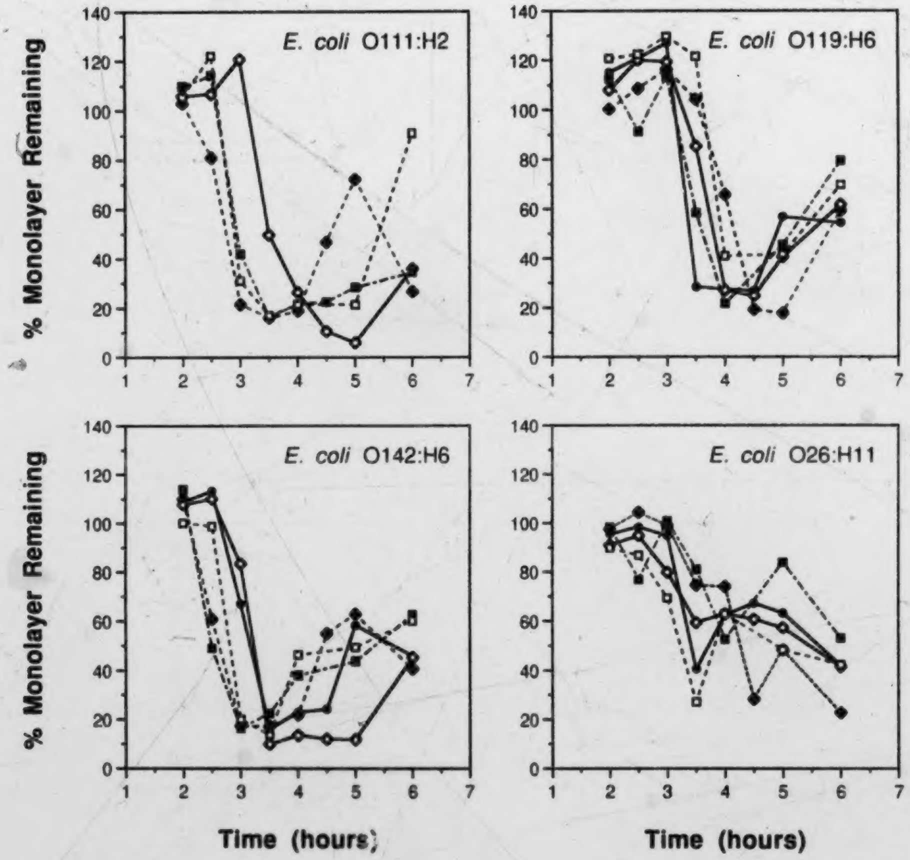

E. coli O111:H2
E. coli O119:H6
E. coli O142:H6
E. coli O26:H11
E. coli O157:H7
E. coli O125:H4
E. coli O126:H27
% Monolayer Remaining
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
Time (hours)

Figure 2.2. Rate of CHO cell monolayer loss caused by EPEC strains (O111:H2, O119:H6, and O142:H6) and non-EPEC O26:H11. Individual lines in each of the panels illustrate the CHO cell response for independent experiments for each bacterial strain: trial 1 (---□---), trial 2 (---■---), trial 3 (—◇—), trial 4 (----◆----), and trial 5 (—◆—). Experimental details are given in the legend to Figure 2.1.


E. coli O111:H2
E. coli O119:H6
E. coli O142:H6
E. coli O26:H11
% Monolayer Remaining
% Monolayer Remaining
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
Time (hours)
Time (hours)

required for transferring *N*-acetylglucosamine to α-1,3-linked mannose exclusively in asparagine-linked (N-linked) glycans on cell surface glycoproteins (39). Consequently, Lec1 cells lack the *N*-acetylglucosamine termini which are required for completing the biosynthesis of sialo (αNeu5NAc(2-6,3)βGal(1-4,3)βGlcNAc) and asialo (βGal(1-4,3)βGlcNAc) lactosamine sequences. Lec2 cells are unable to translocate CMP-sialic acid across Golgi vesicle membranes and are deficient in incorporating sialic acid into glycan sequences on both glycoproteins and glycolipids (10). Lec8 mutants are unable to translocate UDP-galactose into the lumen of the Golgi apparatus and are deficient in galactose incorporation into glycans on glycoproteins and glycolipids (9). Therefore, Lec8 cells lack terminal sialic acid in addition to galactose (sialic acid acceptors) on glycoprotein and glycolipid oligosaccharide sequences. The rate of EPEC-mediated detachment of Lec1, Lec2, or Lec8 cell monolayers was not as rapid as that observed with wild-type CHO cells (Figure 2.3.).

In order to ensure that the absence of rapid monolayer detachment observed using Lec cell lines was not due to the different media used to culture CHO and Lec cells (F-12 nutrient mixture [Ham] and MEM [α medium] respectively), experiments were repeated using Lec2 cells cultured in F-12 nutrient mixture (Ham). EPEC were unable to induce rapid loss of Lec2 cell monolayers grown in the same media as wild-type CHO cells (Figure 2.4.). Furthermore, there was no significant difference in EPEC attachment ($P > 0.593$) to CHO and Lec2 cells cultured in this medium.

2.3.2. Cytotoxins produced by *E. coli* strains. Enterohemolysin or Shiga-like toxins (SLTs) were not responsible for loss of the CHO cell monolayers since neither

Figure 2.3. Alteration of CHO (—□—), Lec1 (·····■·····), Lec2 (- - -◆- - -), and Lec8 (- - -◇- - -) cell adherent properties after co-cultivation with EPEC. Following incubation of the monolayers with bacteria for various times, the cells remaining attached to the tissue culture wells were fixed and stained with Giemsa stain. Stained monolayers were lysed with 2% SDS, and the A_{620}s of these solutions were used to calculate the percent monolayer remaining relative to uninfected monolayers.

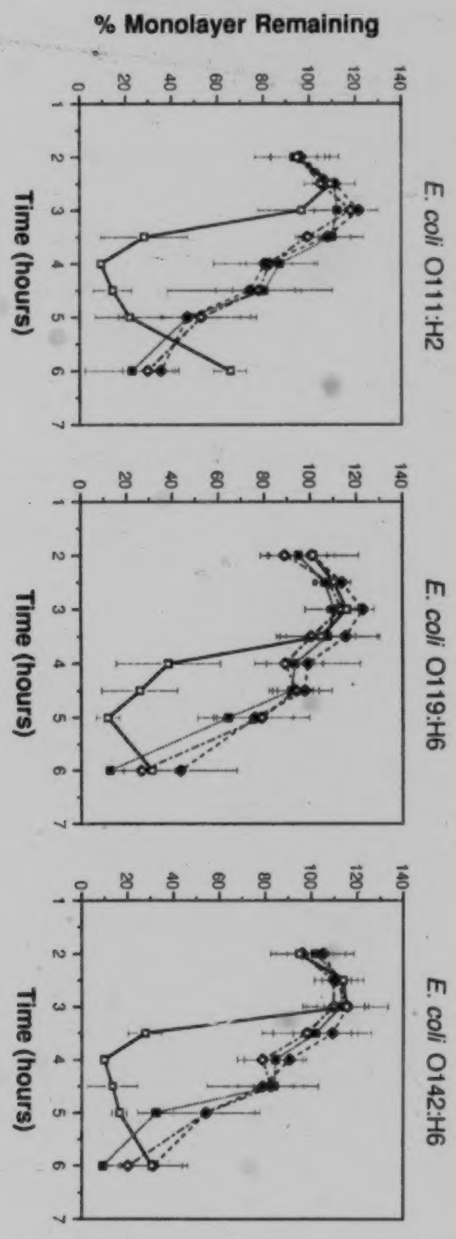
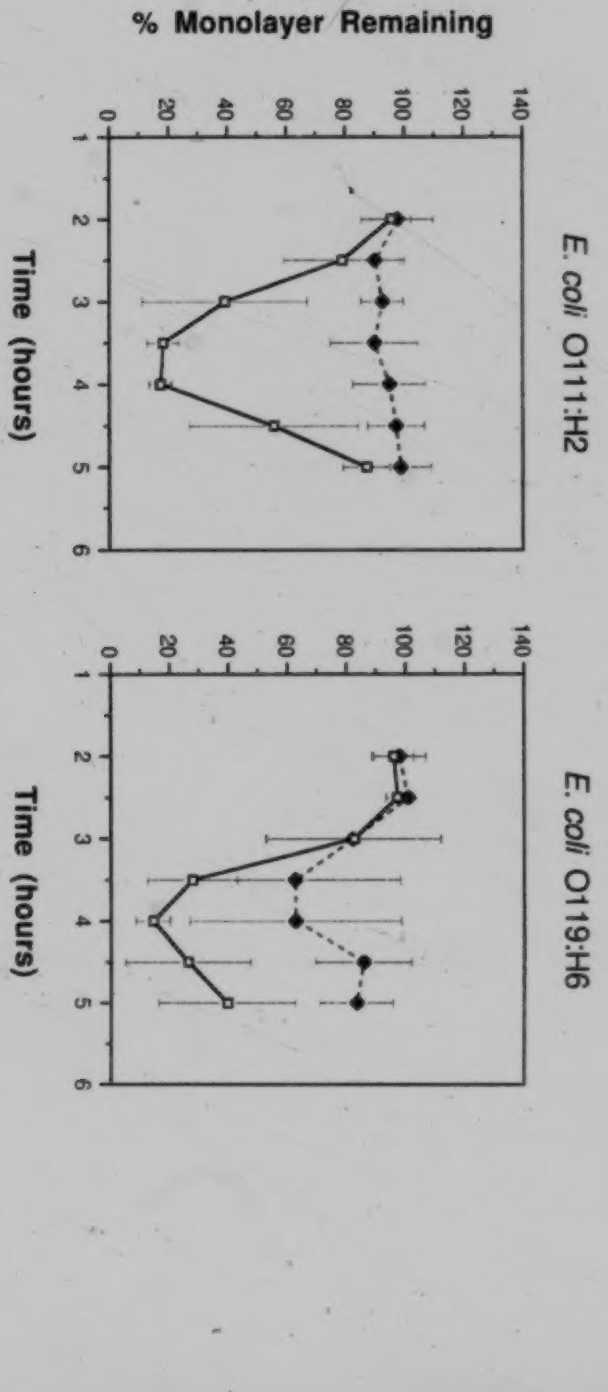

% Monolayer Remaining
E. coli O111:H2
E. coli O119:H6
E. coli O142:H6
0
20
40
60
80
100
120
140
1
2
3
4
5
6
7
Time (hours)

Figure 2.4. EPEC-induced detachment of CHO (—□—) and Lec2 cells (---◆---) cultured in F-12 nutrient mixture (Ham). Lec2 cells were cultured in F-12 nutrient mixture for a minimum of 10 passages before use. Monolayer detachment assays were performed as described in Materials and Methods.


% Monolayer Remaining
E. coli O111:H2
E. coli O119:H6
0
20
40
60
80
100
120
140
1
2
3
4
5
6
Time (hours)
Time (hours)

O157:H7 nor O26:H11 *E. coli*, both of which produce enterohemolysin and Shiga-like toxins, (Table 2.1.) caused a rapid loss of the monolayers similar to that caused by EPEC. Furthermore, we consider it unlikely that the alteration of CHO cell adherent properties was due to the production of cytolethal distending toxin (CDT) (25) by EPEC since cytotoxic effects caused by CDT require 96 to 120 h for observation. In contrast, loss of CHO cell monolayers in our study occurred after 2 h of incubation.

2.3.3. Bacterial replication in tissue culture medium. To determine if the loss of CHO cell monolayers was due to more rapid replication of the EPEC, the growth of bacteria in tissue culture medium was determined. However, no significant differences ($P > 0.068$) were detected in the growth of any of the strains in tissue culture medium (data not shown; [Appendix A, Figure 2.7.]). Therefore, the loss of the monolayer was not due to more rapid growth of the EPEC.

2.3.4. Adherence characteristics of EPEC. Only EPEC, as identified by both their possession of the EAF plasmid and their positive reaction in the FAS test (Table 2.1.), caused the rapid detachment of CHO cell monolayers. These isolates were found to possess both *eae* gene and EAF plasmid sequences which may also be used to identify EPEC. Although *E. coli* O26:H11 and O157:H7 also possess *eae* gene sequences, EPEC EAF sequences were not detected in these serotypes. *E. coli* O26:H11 also caused actin accumulation at sites of bacterial attachment to CHO cells. However, parallel experiments in which adherent bacteria were stained with Giemsa stain demonstrated that very few *E. coli* O26:H11 adhered to CHO cells as compared with the EPEC isolates. It is not known, therefore, if the inability of *E. coli* O26:H11 to cause a rapid detachment of CHO cell monolayers was the result of lower numbers of adherent bacteria or if the rapid loss of the monolayers is an effect unique to EPEC. A positive FAS result was not observed for the *E. coli* O157:H7 isolate because these bacteria completely failed to adhere to CHO cells.

2.3.5. EPEC LA in Lec cells. There was no significant difference ($P = 0.314$) in LA of EPEC strains to Lec2 or wild-type CHO cells (Table 2.2.). This indicated that, although glycan sequences terminating in sialic acid were important for mediating changes in CHO cell adherent properties, LA does not require sialylated glycans. However, significantly fewer Lec1 ($P = 0.008$) and Lec8 ($P = 0.008$) cells had localized adherent bacteria. These results suggest lactosamine sequences are required for expression of LA in CHO cells. Moreover, Lec1 mutants, which express altered N-linked glycan but not O-linked glycan or glycolipid sequences, bound significantly more ($P = 0.011$) EPEC than did Lec8 cells, which have defects in both glycoprotein and glycolipid sequences. Therefore, lactosamine sequences in both glycoproteins and glycolipids appear to play a role in LA of EPEC to CHO cells.

2.3.6. EPEC interaction with CHO and Lec2 cells. Actin accumulation caused by *E. coli* O119:H6 was similar in CHO cells and Lec2 cells (Figure 2.5.). Comparable results were seen using EPEC O111:H2 and O142:H6 (data not shown). These results suggest that sialic acid residues in glycoproteins or glycolipids on CHO cells are not required for EPEC-induced actin accumulation in these cells. EPEC-induced actin accumulation in Lec1 or Lec8 cells was not observed, probably resulting from the poor attachment of the bacteria to these cells. Further, there was no significant difference in the ability of *E. coli* O111:H2 ($P = 0.715$), O119:H6 ($P = 0.225$), or O142:H6 ($P= 0.144$) to invade CHO or Lec2 cells (Figure 2.6.). Therefore, sialic acid residues also do not appear to play a major role in EPEC invasion of CHO cells.

Table 2.2. Percent CHO and Lec Cell Mutants with Localized Adherent EPEC

E. coli Strain	% Cells with Localized Adherent EPEC[a]			
	CHO	Lec1	Lec2	Lec8
O111:H2	86.2 ± 3.6	15.6 ± 11.7	91.5 ± 4.1	9.6 ± 5.6
O119:H6	89.1 ± 4.8	17.7 ± 12.4	88.4 ± 2.7	4.4 ± 1.9
O142:H6	88.7 ± 1.8	15.5 ± 10.9	89.1 ± 4.6	5.7 ± 3.5

[a] Mean ± standard deviation of 3 independent experiments.

Figure 2.5. *E. coli* O119:H6-induced actin accumulation in CHO (A), Lec1 (B), Lec2 (C), and Lec8 (D) cells. After *E. coli* O119:H6 was incubated with tissue culture monolayers in the presence of 0.5% D-mannose for 2 h, the cells were permeabilized with 0.1% Triton-X100 and then stained with FITC-phalloidin. Monolayers were viewed with a fluorescence microscope in order to detect actin accumulation in tissue culture cells.

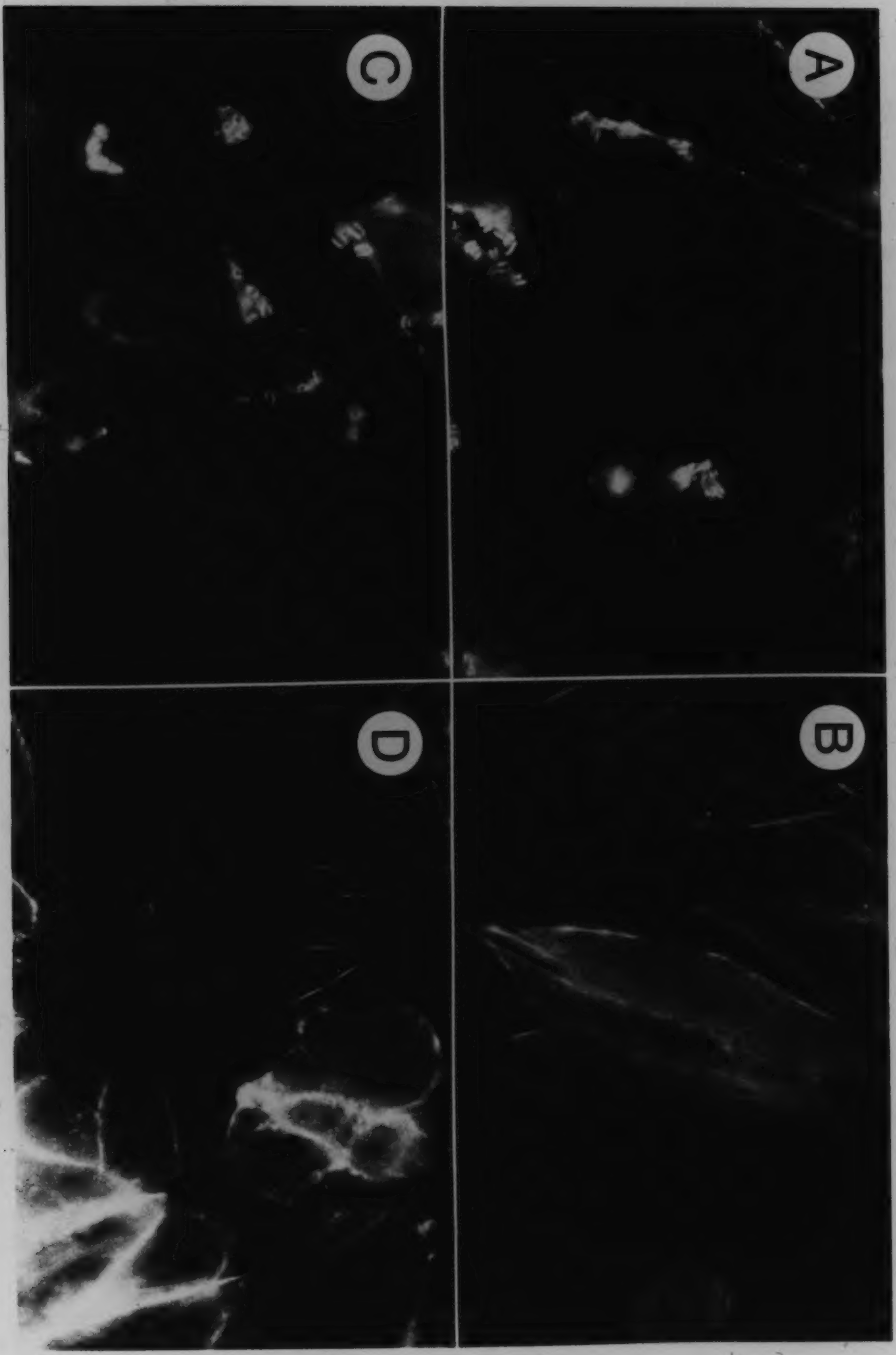
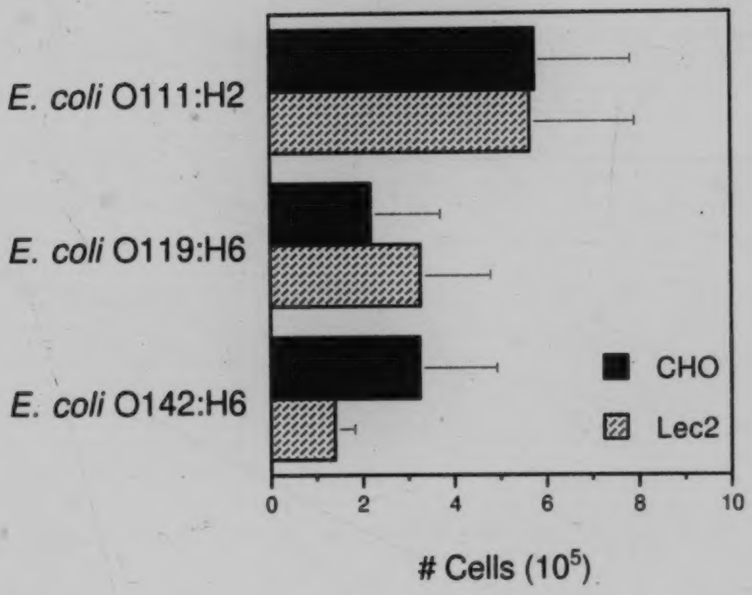

A
B
C
D

Figure 2.6. EPEC invasion of CHO and Lec2 cells. Following incubation of *E. coli* O111:H2, O119:H6, and O142:H6 with CHO or Lec2 cells for 1.5 h in the presence of 0.5% D-mannose, the monolayers were incubated with gentamicin for 1 h and then lysed with 1% Triton X-100. Lysed cell suspensions were diluted in TSB and serial dilutions were performed to determine the number of invasive bacteria.



E. coli O111:H2
E. coli O119:H6
E. coli O142:H6
CHO
Lec2
0
2
4
6
8
10
# Cells (10^5)

2.4. DISCUSSION

According to the proposed model for EPEC pathogenesis (11, 12), EPEC attachment to epithelial cells is a multistep process. Initially, EPEC adhere to epithelial cells in a characteristic, LA pattern. Subsequently, intimate interaction occurs resulting in cytoskeletal rearrangement within the enterocyte. This is followed by EPEC invasion of the eukaryotic cell. These last processes are accompanied by an increase in intracellular calcium levels (4, 13) and protein phosphorylation (3, 30, 35). Although the pharmacological effects of EPEC interaction with eukaryotic cells are becoming better understood, the biochemical nature of the receptors involved remains to be determined. Moreover, it is still uncertain whether attachment and invasion of eukaryotic cells is sufficient to cause all the clinical symptoms associated with EPEC infections.

Results published previously by Rafiee *et al.* demonstrated that AF/R1 pili of RDEC-1, an enteropathogenic *E. coli* strain of rabbits, bind to sialated glycoprotein complexes in rabbit ileal microvillus membranes (34). Lec1 cells, which were used in our experiments, lack N-acetylglucosaminyltransferase (GlcNAc-T1) activity, which results in the expression of oligosaccharide structures terminating in mannose on the surface of these cells (39). O-linked oligosaccharides and glycolipids in this mutant are the same as those present in wild-type CHO cells. In our experiments, the significant reduction in the number of Lec1 cells having locally adherent EPEC suggested that sialyllactosamine in glycoprotein sequences on CHO cells are important for the initial bundle-forming pilus-mediated attachment of EPEC (18) (Table 2.2.). However, no significant difference was observed between EPEC LA to wild-type CHO cells or Lec2 CHO cell mutants, which are deficient only in sialic acid incorporation into glycoproteins and glycolipids (10). Therefore, in contrast to the findings of Rafiee *et al.* (34) using rabbit EPEC, our data suggest sialic acid groups are not required for LA of human EPEC in CHO cells.

The recognition of asialo glycans on CHO cells by EPEC in our study is consistent with results published by Jagannatha *et al.* (21) indicating that EPEC bound to asialo-glycolipids immobilized on thin layer chromatography plates. Lec8 cells, which are defective in both glycoprotein and glycolipid biosynthesis (9), were more impaired than Lec1 (defective only in N-linked glycans on glycoproteins) cells in their ability to bind EPEC (Table 2.2.). This suggested that oligosaccharide sequences in glycolipids also play a role in EPEC attachment.

The role of sialated sequences in signal transduction processes following intimate attachment by EPEC was examined using CHO and Lec2 cell mutants. There was no observable difference in actin accumulation caused by any of the three EPEC strains (O111:H2, O119:H6, and O142:H6) in either CHO or Lec2 cells. Also, there were no significant differences in the numbers of EPEC which invaded CHO or Lec2 cells. Further investigation is required to determine whether mechanisms responsible for CHO monolayer detachment are distinct from signal transduction processes associated with EPEC attachment and invasion (i.e. intracellular calcium mobilization and protein phosphorylation).

When grown at 37°C pathogenic *Yersinia* spp. express two plasmid-encoded proteins, YopE and YopH, that are cytotoxic for HEp-2 cells and mouse macrophages (6, 7, 19, 33, 37). Further, intimate attachment of yersiniae to the host cell membrane is apparently essential for functional expression of YopE and YopH cytotoxicity (33, 36). The activity of these *Yersinia* virulence factors can be monitored by monolayer detachment assays similar to the one described in this article.

Although we do not, as yet, have any data, we are tempted to speculate that EPEC, like yersiniae, may express other virulence factors in addition to those involved in attachment and invasion of eukaryotic cells. These do not appear to be enterohemolysin or

Shiga-like toxins as none of these toxins were detected in the EPEC strains used in our experiments. Further, the EPEC factor(s) responsible for CHO cell detachment may not be cytotoxic since the effect appeared to be partially reversible within the time frame of our experiments (Figures 2.1. and 2.2.).

Our results demonstrated that EPEC strains failed to cause rapid detachment of Lec2 cell monolayers similar to that seen with wild-type CHO cells. EPEC attachment to and invasion of CHO cells does not appear to be directly responsible for CHO cell detachment since these processes were normal in Lec2 mutants. Our laboratory is currently investigating the biochemical basis for EPEC-mediated detachment of CHO cell monolayers with the objective of revealing additional virulence mechanisms in these complex intestinal pathogens. The CHO cell detachment assay will be useful in obtaining EPEC mutants that are defective in the expression of this phenotype.

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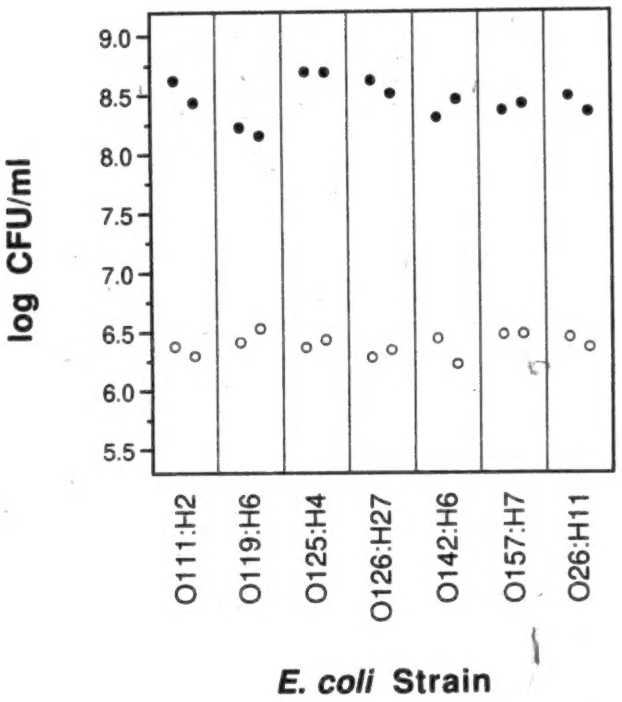
2.6 APPENDIX A

Figure indicated as "data not shown" in the text.

Figure 2.7. Determination of the number of colony forming units (CFU) of various *E. coli* strains after growth in tissue culture medium. Log phase TSB-grown organisms were added to F-12 nutrient mixture (Ham) and at 0 hours (○) or after 3 hours incubation at 37°C in a CO_2 incubator (●), an aliquot of the culture was removed and serially diluted to determine the number of CFU.


log CFU/ml
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
O111:H2
O119:H6
O125:H4
O126:H27
O142:H6
O157:H7
O26:H11
E. coli Strain

CHAPTER 3

Role of Lactosyl Glycan Sequences in Inhibiting Enteropathogenic *Escherichia coli* Attachment

3. PREFACE

In order to support our results that enteropathogenic *Escherichia coli* (EPEC) bound to lactosamine sequences on Chinese hamster ovary cells, a second approach was used to identify sequences recognized by EPEC. For these experiments, synthetic, multivalent bovine serum albumin-glycoconjugates were used to compete for EPEC binding to epithelial cells (3). This approach offered the advantages of examining EPEC binding to intact epithelial cells using glycoconjugates which were more representative of the interactions between microbial adhesin(s) and receptors on eukaryotic cells (2, 4). By comparing the relative abilities of specific oligosaccharide structures to inhibit binding, I could also examine the roles of additional sugar residues in attachment (1).

During my investigations into the effectiveness of various glycoconjugates as inhibitors of EPEC binding, I observed that the pre-incubation of bacteria with a particular glycoconjugate increased its effectiveness as an inhibitor. Based on this observation, additional experiments were performed to determine whether interaction of EPEC with the glycoconjugates altered the bacteria in a way that reduced their ability to bind.

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3.1. INTRODUCTION*

Enteropathogenic *Escherichia coli* (EPEC) is a significant cause of diarrhea in young children, especially those less than 6 months of age (7, 54). Clinical symptoms are associated with intestinal attaching and effacing (A/E) lesions which are characterized by the degeneration of microvilli, and the intimate association of bacteria with pedestal-like structures formed from host epithelial cells (3, 36, 42, 50). Donnenberg and Kaper originally proposed that EPEC colonization of intestinal epithelial surfaces occurs by a three-stage process (10). According to this multi-step model, the initial binding of EPEC to intestinal epithelial cells involves their non-intimate attachment as microcolonies. Next, the bacteria secrete several proteins which induce signal transduction pathways in epithelial cells. These signals initiate cytoskeletal rearrangement and the effacement of microvilli of host cells. In the final stage of attachment, the cytoskeletal components are organized to form pedestal-like structures which partially surround adherent organisms. More recently, Hicks *et al.* proposed a modified model for EPEC colonization (18). This model is similar to the three stage model of Donnenberg and Kaper except that three-dimensional microcolonies are thought to develop after, not before, intimate attachment.

Since adherence is an important factor in EPEC pathogenesis, considerable research has been performed to identify bacterial and eukaryotic cell structures involved in attachment. So far, two bacterial structures have been relatively well characterized. The first, bundle-forming pili (BFP), are associated with the initial, non-intimate attachment of EPEC as microcolonies to discrete sites on epithelial cells, a pattern which is referred to as localized adherence (LA) (4, 16, 44). Scanning electron micrographs of LA EPEC revealed that BFP are involved in mediating inter-bacterial linkages within microcolonies. Whether the BFP also function as adhesins for EPEC binding to epithelial cells remains to be resolved, however, since these structures appear to mediate bacterial binding to HEp-2 cells but not to human intestinal tissue in organ culture (16, 18, 48). A second bacterial protein involved in attachment is intimin (22, 23). This protein is necessary for a later stage of EPEC attachment in that it focuses host cell cytoskeletal components beneath adherent bacteria to form A/E lesions (39).

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In contrast to what is known about bacterial structures involved in attachment, EPEC receptors on host eukaryotic cells are less well characterized. Rosenshine *et al.* originally identified a 90 kDa tyrosine-phosphorylated protein (Hp90) in epithelial cell membranes which bound intimin (41). This protein was subsequently shown to be a secreted bacterial protein, called Tir (translocated intimin receptor), which, after translocation into the eukaryotic cell, becomes phosphorylated and serves as the receptor for intimin (6, 25). Frankel *et al.* reported that intimin may also bind to β_1 integrins (14).

In addition to these reports, several groups have identified oligosaccharide structures that are potential receptors for EPEC. Using various experimental approaches, *N*-acetylgalactosamine (43), fucosylated milk oligosaccharides (5), GM_3 gangliosides (19) and the GalNAc$\beta(1\rightarrow4)$Gal portion of asialo-GM_1 and asialo-GM_2 structures (20) have all been implicated in EPEC attachment to eukaryotic cells. As well, we previously reported that EPEC bound to asialo-lactosamine sequences of N-linked glycoproteins on Chinese hamster ovary (CHO) cells (52). A possible role for O-linked glycoproteins or glycolipids in EPEC attachment to CHO cells was also suggested by data presented in our previous report.

In general, several reports suggest that EPEC recognize lactosyl structures on epithelial cells. However, additional carbohydrate groups (i.e., sialic acid and fucose) are frequently attached to these core structures. While our previous results using CHO cell Lec mutants indicated that EPEC do not require sialic acid in order to bind, the importance of fucose in these interactions was not addressed since CHO cells do not express certain fucosylated glycans (38). In consideration of previous results which suggested that fucosylated structures are preferentially recognized by EPEC (5), we performed binding inhibition experiments with synthetic lactosamine or fucosylated lactosamine BSA-glycoconjugates, to investigate the role of fucose in EPEC attachment to host cells. Our results demonstrated that *N*-acetyllactosamine (LacNAc)-BSA, followed by Lewis X (LeX)-BSA, were the most effective soluble inhibitors of EPEC E2348/69 attachment to HEp-2 cells. Furthermore, the interaction of bacteria with these specific glycoconjugates, alone, caused a decrease in the expression of BfpA, the structural subunit of BFP (9, 46), and intimin, in these organisms. These results suggest that EPEC may possess a mechanism for regulating the expression of these proteins which could contribute to the reduced ability of the bacteria to bind to epithelial cells.

3.2. MATERIALS AND METHODS

3.2.1. Reagents. All glycoconjugates consisted of chemically synthesized oligosaccharide sequences (Table 3.1.) covalently conjugated to bovine serum albumin (BSA) through an 8-methoxycarbonyloctyl linker arm (33). LacNAc-BSA and Lewis Y (LeY)-BSA were generously provided by O. Hindsgaul (University of Alberta, Edmonton,

Table 3.1. Oligosaccharides Sequences of BSA-Glycoconjugates

Oligosaccharide	Structure
N-acetyllactosamine	Galβ(1→4)GlcNAc
Lewis X	Galβ (1→4)[Fucα (1→3)]GlcNAc
Lewis Y	Fucα (1→2) Galβ (1→4)[Fucα (1→3)] GlcNAc

Alberta, Canada). LeX-BSA was purchased from the Alberta Research Council (Edmonton, Alberta, Canada). The incorporation of ligands into BSA (mol/mol) was determined by mass spectroscopy to be as follows: LacNAc-BSA (19:1), LeX-BSA (26:1) and LeY-BSA (17:1). All glycoconjugates were solubilized in phosphate-buffered saline (PBS) (5 mg/ml) and stored at -20°C prior to use. Polyclonal rabbit anti-intimin (22) and anti-BfpA (56) antibodies were kindly provided by J. B. Kaper and M. S. Donnenberg (University of Maryland School of Medicine, Baltimore, Md.), respectively. Rabbit anti-maltose-binding protein (MBP) antibodies were purchased from New England Biolabs (Mississauga, Ontario, Canada).

3.2.2. Bacterial strains. EPEC strain E2348/69 (O127:H6), which is a wild-type strain isolated from an infant with diarrhea (34), was generously provided by B. B. Finlay (University of British Columbia, Vancouver, British Columbia, Canada). In each experiment, bacteria which were stored as frozen stock cultures at –70°C, were grown overnight at 37°C on tryptic soy agar (Difco, Detroit, Mich.) plates. An isolated bacterial colony was then inoculated into tryptic soy broth (TSB) and incubated for 16 h, without shaking, at 37°C under normal atmospheric conditions for use in experiments the following day.

3.2.3. Preparation of HEp-2 cell monolayers. HEp-2 cells (CCL-23) were obtained from the American Type Culture Collection (Rockville, Md.). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air in minimal essential medium supplemented with 10% fetal bovine serum (FBS). Sub-confluent monolayers were prepared for the binding assays by disrupting HEp-2 monolayers with a solution of 0.25% (vol/vol) tissue culture grade trypsin in FC buffer (0.14 M NaCl, 5.0 M mM KCl, 20.0 mM Tris-HCl, 5.0 mM Tris base, 0.5 mM EDTA [pH 7.2]). After suspending the trypsinized HEp-2 cells in fresh tissue culture medium, approximately 7.5 x 10^3 cells in 150 µl of culture medium were added to individual wells, each well containing a 6 mm-diameter removable polystyrene disk (Biomedical Workshop, University of Alberta, Edmonton, Alberta, Canada) covering their bottoms, of 96-well tissue culture plates. The plates were then incubated in a CO_2 incubator until the next day.

3.2.4. EPEC cell binding assay. For all experiments, EPEC E2348/69 was cultured in Dulbecco's modified Eagle medium (DMEM) (catalog no. 23800; Gibco, Burlington, Ontario, Canada) supplemented with 44 mM $NaHCO_3$, 40 µM phenol red, and 25 mM glucose, which was pre-equilibrated overnight in a CO_2 incubator (51). FBS was not included in this culture medium. Prior to each experiment, 40 µl of the TSB-grown bacteria were inoculated into 4 ml DMEM in 15 x 75 mm borosilicate glass culture tubes which were then incubated for 1 h in a CO_2 incubator to induce the expression of EPEC virulence factors (40, 51, 53). To determine the optimum concentration of glycoconjugates for the binding inhibition experiments, 32 µl of DMEM bacterial culture (approximately 3-5x10^6 colony forming units), and 52 µl DMEM (pre-equilibrated) were added to empty wells of a 96-well microtiter plate. Next, 16 µl of BSA-glycoconjugate solution (undiluted [5 mg/ml], or diluted 1:2 or 1:4 in PBS) was added to the wells to obtain final inhibitor concentrations of 0.8, 0.4, or 0.2 mg/ml, respectively. For all subsequent experiments, the volumes were adjusted such that 32 µl of DMEM bacterial culture, 56 µl DMEM and 12 µl of BSA-glycoconjugate (final concentration of 0.6 mg/ml) were added to wells of a 96-well plate. After mixing the contents of each well, the plate was incubated, without agitation, for 30 min at 37°C in a CO_2 incubator. The entire contents from each of the wells were then transferred to wells containing sub-confluent HEp-2 cell monolayers from which the culture medium was first removed. This microtiter plate was incubated for an additional 30 min at 37°C in the CO_2 incubator, after which time the cells were washed three times with PBS, fixed with methanol for 10 min, and stained with Giemsa stain for 20 min. The polystyrene disks were removed from the microtiter plate wells and EPEC adherence was monitored microscopically using the 100x objective lens. Depending on the number of microscopic fields required to view the entire area of the coverslip, 150 –200 randomly chosen HEp-2 cells were examined. Those having attached microcolonies consisting of more than 4 bacteria were considered positive for LA EPEC (53). The number of replicates for each sample of a trial is indicated in the legend for each figure.

3.2.5. Effect of glycoconjugate pre-incubation period on bacterial attachment. EPEC E2348/69 was grown in DMEM for 1 h in a CO_2 incubator as described above. Next, 32 µl of bacterial culture and 56 µl DMEM were added to empty wells of a 96-well tissue culture plate. At this time, 12 µl BSA-glycoconjugate (final concentration of 0.6 mg/ml per well) was added to sample wells for which the effect of pre-incubation with the BSA-glycoconjugate(s) was to be determined. After mixing the contents of each well, the plate was placed in a CO_2 incubator for 30 min. Next, 12 µl of

BSA-glycoconjugate was added to the remaining, no pre-incubation, sample wells of the plate. The contents of all wells were then immediately transferred to wells of a 96-well microtiter plate containing sub-confluent HEp-2 cell monolayers grown on polystyrene disks, and the plate was incubated, without agitation, in a CO_2, 37°C incubator for 30 min. The cells were then washed three times with PBS, fixed with methanol and stained with Giemsa stain. The percentage of HEp-2 cells with LA EPEC was determined as described in the previous section.

3.2.6. Determination of BfpA and intimin expression. After incubating EPEC E2348/69 in DMEM for 1 h to induce the expression of virulence factors, 96 µl of bacterial culture, 168 µl of DMEM and 36 µl of BSA-glycoconjugate (final concentration 0.6 mg/ml per well) were added to empty wells of a 48-well tissue culture plate. The plate was incubated in a CO_2 incubator for 1 h as described earlier, before transferring the contents of each well to microcentrifuge tubes. The bacteria were washed once with PBS by centrifugation and the resulting pellet was lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM dithiothreitol (31). Bacterial proteins were separated by SDS-PAGE (12.5% polyacrylamide) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.).

Since different antibodies were used to detect specific proteins, the membrane was cut into 3 sections using the pre-stained molecular size standards as a guide. Non-specific binding sites of the membranes were blocked with 5% skim milk (wt/vol) in PBS containing 0.05% Tween-20 (PBST), and the sections were incubated with various antibodies according to the recommendations of the supplier. The dilution for each antibody was as follows: anti-intimin (1:2000), anti-MBP (1:10,000) and anti-BfpA

(1:4000). After incubation with the antibodies for 2 h at room temperature, the membranes were washed three times with PBST and then incubated with goat anti-rabbit peroxidase-conjugated antibodies (1:18,000 dilution) for 1.5 h at room temperature. The membranes were washed again with PBST, followed by three washes with PBS, and finally incubated with the enhanced chemiluminescence (ECL) color development reagents according to the manufacturer's instructions (Amersham, Oakville, Ontario, Canada). Protein bands were visualized by exposing the membranes to Kodak X-Omat blue XB-1 film. Bands corresponding to intimin, MBP and BfpA in each sample were analyzed using an LKB Ultroscan XL laser densitometer supplied with an LKB 2220 integrator [Appendix A, Figure 3.6.]. MBP was chosen as an internal standard since the level of this protein was not expected to vary between samples (15). The relative amounts of intimin and BfpA in each sample were determined by normalizing the levels of these proteins to the amount of the internal standard, MBP, in each gel lane (i.e. $\text{Area}_{\text{intimin}}/\text{Area}_{\text{MBP}}$ or $\text{Area}_{\text{BfpA}}/\text{Area}_{\text{MBP}}$, respectively).

3.3. RESULTS

3.3.1. Inhibition of EPEC LA by the BSA-glycoconjugates. To identify possible carbohydrate sequences involved in EPEC E2348/69 binding to HEp-2 cells, we first cultured the bacteria in tissue culture medium to induce the expression of virulence factors. These bacteria were then pre-incubated with a specific glycoconjugate (Table 3.1.) for 30 min at 37°C. Finally, the EPEC-glycoconjugate mixtures were added to HEp-2 cell monolayers for an additional 30 min to allow bacterial binding to occur.

Preliminary experiments were performed with various glycoconjugate concentrations. The results of these dose-dependent binding inhibition experiments demonstrated that LacNAc-BSA was the most effective inhibitor of EPEC E2348/69 attachment to HEp-2 cells (Figure 3.1.). This inhibition was concentration dependent over the range examined, 0.2, 0.4, or 0.8 mg/ml of LacNAc-BSA, resulting in approximately 40%, 65%, and 87% reduction, respectively, in EPEC LA to HEp-2 cells.

Based on the inhibitory effects observed with LacNAc-BSA in Figure 3.1., we used the BSA-glycoconjugates at a final concentration of 0.6 mg/ml in all subsequent experiments. To confirm the appropriateness of this concentration, three additional experiments were performed using EPEC E2348/69 (Figure 3.2.). Similar to the results presented in Figure 3.1., LacNAc-BSA inhibited EPEC attachment to the greatest extent. LeX-BSA was the second most effective inhibitor of EPEC binding in these experiments, with LeY-BSA being the least effective. Comparable results were observed in later experiments performed in a similar manner (Figure 3.5.). Additional control experiments confirmed that the inhibitory effect of these glycoconjugates was not due to a toxic effect of these compounds on the bacteria (data not shown; [Appendix A, Figure 3.7.]).

3.3.2. Effect of pre-incubation on EPEC attachment. The procedure used in the glycoconjugate inhibition binding experiments involved pre-incubating EPEC E2348/69 with various glycoconjugates for 30 min prior to adding the mixtures to the HEp-2 cell monolayers. This pre-incubation step was performed on the assumption that the multivalent BSA-glycoconjugate inhibitors required a finite amount of time to occupy the maximum number of bacterial adhesin receptor binding sites prior to exposing the EPEC-glycoconjugate mixtures to the HEp-2 cells. This assumption would predict that the BSA-glycoconjugate inhibitors might be less effective if this pre-incubation step was omitted. To test this prediction, experiments were performed in which the bacteria were either pre-incubated for 30 min with LacNAc-BSA before being added to the HEp-2 cells, or were added simultaneously with LacNAc-BSA to the monolayers. As shown in Figure 3.3.,

Figure 3.1. Optimization of BSA-glycoconjugate(s) concentration for experiments. After culturing EPEC E2348/69 in DMEM for 1 h in a CO_2 incubator, the bacteria, and various concentrations of BSA-glycoconjugate, were added to wells of a 96-well plate. The plate was then incubated in a CO_2 incubator for 30 min, after which time the contents of each well were transferred to wells of a 96-well plate containing subconfluent monolayers of HEp-2 cells grown on polystyrene coverslips. After incubating this plate in a CO_2 incubator for an additional 30 minutes, the cells were washed with PBS, fixed with methanol and stained with Giemsa stain. HEp-2 cells with adherent microcolonies consisting of five or more bacteria were considered positive for LA EPEC. Each data point represents the average of single determinations for a sample obtained in two independent trials. Error bars indicate the range in the data for each sample from both trials. BSA (—●—), LacNAc-BSA (—△—), LeX-BSA (•••○•••), and LeY-BSA(┈┈▲┈┈).

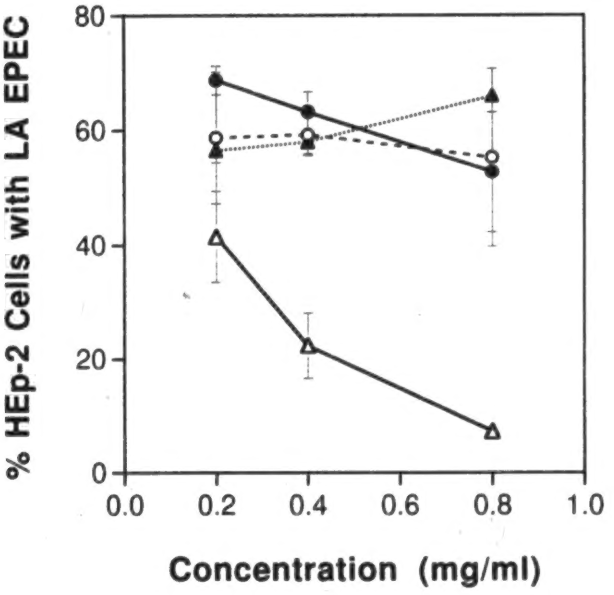

% HEp-2 Cells with LA EPEC
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
Concentration (mg/ml)

Figure 3.2. Inhibition of EPEC E2348/69 binding to HEp-2 cells using BSA-glycoconjugates at a final concentration of 0.6 mg/ml per well. Experiments were performed essentially as described in the legend to Figure 3.1. except that bacteria were incubated with BSA-glycoconjugates present at final concentrations of 0.6 mg/ml per well. After allowing the bacteria to bind to HEp-2 cells, the cells were washed with PBS, fixed with methanol and stained with Giemsa stain. The percentage of HEp-2 cells with LA EPEC was determined as described earlier. Data points for Trial 1 (●), Trial 2 (▲) and Trial 3 (○) are indicated. Error bars for each of these points represent the range in values obtained for duplicate samples. The over-laying bar graph indicates the mean(s) of the data obtained in all three trials.

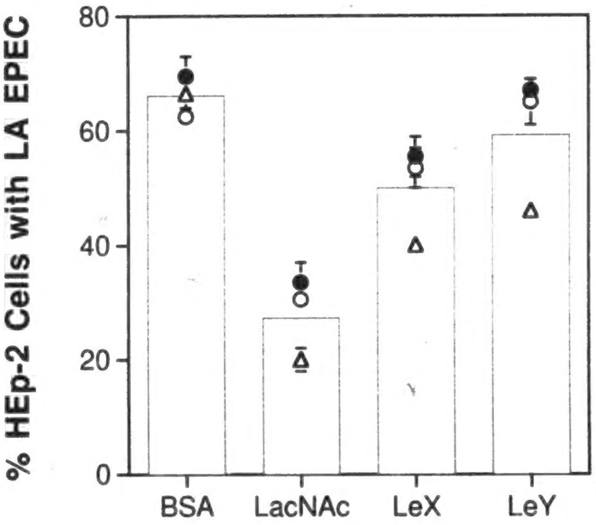
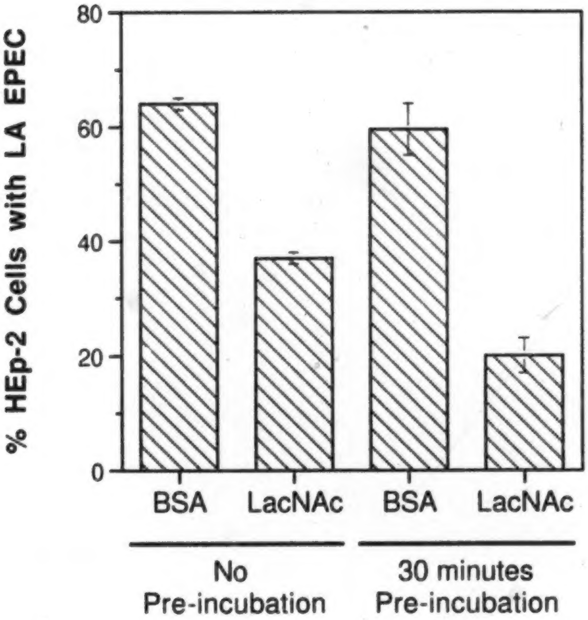

% HEp-2 Cells with LA EPEC
80
60
40
20
0
BSA
LacNAc
LeX
LeY

Figure 3.3. Effect of pre-incubation period on EPEC E2348/69 attachment to HEp-2 cells. Overnight TSB-grown bacteria were grown in DMEM as described earlier. After transferring the bacteria to empty wells of a 96-well plate, LacNAc-BSA (final concentration of 0.6 mg/ml per well) was also added to wells for which the effect of pre-incubation was to be determined. The plate was then incubated in a CO_2 incubator for 30 min, after which time LacNAc-BSA was added to the remaining sample wells. The contents of all wells were immediately transferred to wells containing subconfluent HEp-2 cell monolayers and this plate was incubated in a CO_2 incubator for 30 min. After washing with PBS, the cells were fixed with methanol and stained with Giemsa stain. HEp-2 cells considered positive for LA EPEC were determined as described earlier. The results shown are representative of those obtained in three independent experiments. The error bars represent the range in values for duplicate samples of a single trial.


% HEp-2 Cells with LA EPEC
80
60
40
20
0
BSA
LacNAc
BSA
LacNAc
No
Pre-incubation
30 minutes
Pre-incubation

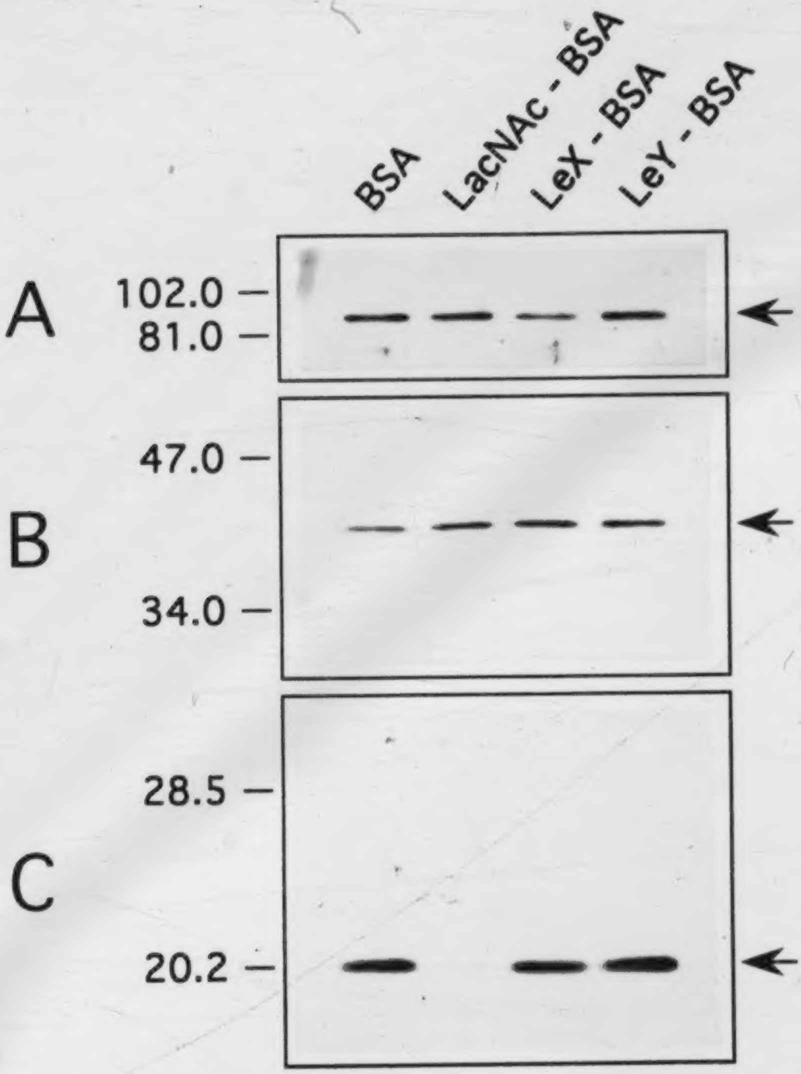
eliminating the pre-incubation step resulted in an approximately two-fold decrease in the inhibitory activity of LacNAc-BSA. Nevertheless, even without the pre-incubation step, LacNAc-BSA reduced EPEC binding to the HEp-2 cells by 42%.

3.3.3. Effect of BSA-glycoconjugates on the expression of EPEC BfpA and intimin. The observation that the effectiveness of LacNAc-BSA as an inhibitor of EPEC E2348/69 binding to HEp-2 cells depended on the time of the pre-incubation step was consistent with the assumption that this inhibitor required a finite amount of time to occupy a majority of the adhesin receptor binding domains. It was also possible, however, that during this preincubation period the LacNAc-BSA caused an alteration in the expression of proteins involved in EPEC adherence. To investigate this possibility, we measured the relative expression of BfpA and intimin of EPEC pre-incubated with different glycoconjugates in parallel with binding inhibition experiments. The results of these experiments demonstrated that the expression of BfpA and intimin were reduced when the bacteria were incubated with specific glycoconjugates (Figure 3.4.). An analysis of the levels of these proteins by densitometry revealed that BfpA and intimin levels, overall, were decreased to the greatest extent by LacNAc-BSA and LeX-BSA, respectively (Figure 3.5.).

3.4. DISCUSSION

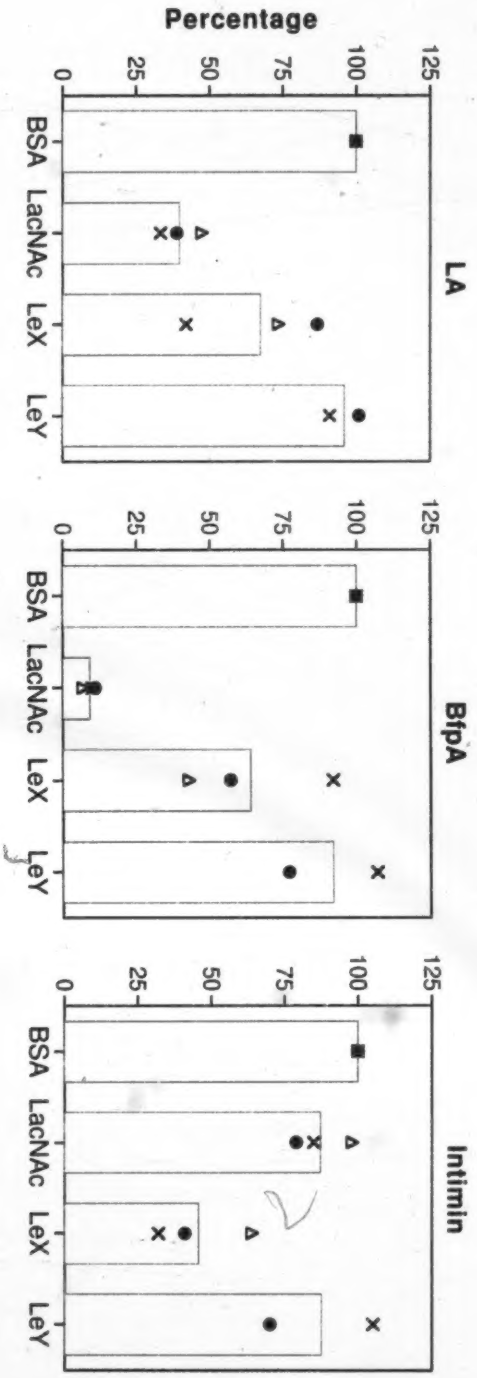
Using CHO cell Lec mutants, we previously demonstrated that the expression of asialo-lactosamine sequences was sufficient for EPEC attachment to these cells (52). In the present study, we used synthetic, multivalent BSA-glycoconjugates presenting either lactosamine (LacNAc) or fucosyllactosamine structures (LeX or LeY) to determine whether fucosylated glycan sequences also had a role in these interactions (5). EPEC E2348/69

Figure 3.4. Effect of BSA-glycoconjugates on the expression of EPEC proteins associated with attachment. EPEC E2348/69 was cultured in DMEM for 1 h in a CO_2 incubator and then transferred to wells of a 48-well plate. After adding BSA-glycoconjugates (0.6 mg/ml final concentration) to these wells, the plate was incubated for an additional 1 h in a CO_2 atmosphere. The contents of each well were then transferred to microcentrifuge tubes, washed with PBS, and lysed in sample buffer. Sample proteins were separated by SDS-PAGE (12.5% polyacrylamide) and transferred electrophoretically to Immobilon-P membrane. Bacterial proteins were detected with (A) anti-intimin, (B) anti-maltose-binding protein or (C) anti-BfpA antibodies followed by anti-rabbit peroxidase-conjugated antibodies and the ECL detection system. The mobilities of pre-stained molecular size standards (in kilodaltons) are indicated on the left while proteins detected using the antibodies are indicated by arrows on the right.



BSA
LacNAc - BSA
LeX - BSA
LeY - BSA
A
102.0
81.0
B
47.0
34.0
C
28.5
20.2

Figure 3.5. Relationship between EPEC binding and BfpA or intimin levels. For each trial, the experiments investigating the effects of various glycoconjugates on bacterial binding and levels of BfpA or intimin were performed in parallel. The assay for EPEC LA to HEp-2 cells was performed as described in the legend to Figure 3.2. Intimin, maltose-binding protein, and BfpA were detected as described in the legend to Figure 3.4. After analyzing each of the bands by densitometry, the levels of BfpA and intimin were normalized to the level of maltose-binding protein in each sample. Percentage (y-axis) for each figure refers to the levels of LA, BfpA or intimin of EPEC incubated with specific glycoconjugate(s) relative to that of bacteria cultured in the presence of BSA. Each data point represents a single determination for Trial 1 (△), Trial 2 (✕) and Trial 3 (●). The over-laying bar graph indicates the mean(s) of the data collected in all trials. LeY-BSA was not used in Trial 1.


Percentage
0
25
50
75
100
125
LA
BfpA
Intimin
BSA
LacNAc
LeX
LeY

was selected for these experiments since this strain is commonly used by other research groups to investigate EPEC pathogenesis (8, 9, 34, 48). Overall, we found that LacNAc-BSA was the most effective inhibitor of bacterial binding to HEp-2 cells, followed by LeX-BSA. LeY-BSA was the least effective inhibitor in our binding assays. These results confirmed our previous findings regarding the involvement of lactosamine sequences in EPEC binding to CHO cells. Additional experiments performed to elucidate the manner in which these glycan structures inhibited binding led to the observation that incubation of EPEC E2348/69 with LacNAc-BSA and LeX-BSA alone caused decreases in the expression of BfpA and intimin, respectively. These results are significant because they revealed that the expression of BfpA and intimin, two different proteins involved in EPEC attachment, were specifically affected by different glycoconjugates.

In previous experiments where carbohydrates were shown to inhibit bacterial attachment to tissue culture cells, it was presumed that these structures were host cell receptor analogs which acted as competitive antagonists of bacterial binding (2, 12). However, the results of our experiments, which indicated that the inhibition of EPEC E2348/69 binding by glycoconjugates correlated with a decrease in the expression of BfpA and intimin, suggested that the process of inhibition may be more complex. Recently, Knutton *et al.* demonstrated that EPEC down-regulated intimin expression in the later stage(s) of attachment to HEp-2 cells (28). A similar decrease in BFP levels was not observed during the same time period. Our results are similar to those of Knutton *et al.* in that we also found that EPEC expressed lower levels of a protein(s) associated with attachment in response to environmental signals. As well, our results and those of Knutton *et al.* suggest that intimin and BFP, to a degree, might be differentially regulated. Unlike their results, however, we found that specific glycoconjugates were able to mediate this effect independent of viable epithelial cells and affected BFP expression as well.

As of yet, we do not know the mechanism by which the glycoconjugates mediate their effect on BfpA and intimin. In consideration of our results and those of Knutton *et al.*, an attractive model is that, in addition to the glycoconjugates possibly acting as competitive inhibitors of binding, their interaction with EPEC may stimulate bacteria to progress to later stages of the multi-step colonization process. Ultimately, this progression to a later stage is accompanied by a loss of structures required for attachment, and this reduces the ability of the bacteria to bind. While additional data is required to support this model, it is interesting to speculate that other virulence factors of EPEC could be similarly affected. Recently, Knutton *et al.* demonstrated that EspA-associated surface structures also disappear from EPEC cell surfaces during the formation of A/E lesions (30). Since this pattern of expression resembles that seen with intimin, it is possible that the secretion of proteins involved in EPEC virulence (6, 17, 21, 25-27, 30, 32) could also be affected by the interaction of bacteria with specific glycoconjugates.

An implication of the above model is that the glycoconjugates are analogs of epithelial cell receptors for EPEC. In our experiments, we used HEp-2 cells which are derived from laryngeal tissue. While results of investigations into the formation of A/E lesions with this cell line appear to parallel *in vivo* findings (29), recent results of Hicks *et al.* suggest that the initial attachment of EPEC to HEp-2 cells differs from that observed with intestinal cells (18). Specifically, Hicks *et al.* reported that EPEC which expressed BFP, but not intimin, were able to attach to HEp-2 cells, but not to intestinal biopsy tissue. Whether LacNAc and LeX sequences used in our experiments mimic actual intestinal cell receptors *in vivo* remains to be determined. However, an important aspect of our results with the soluble BSA-glycoconjugates is that it may not be necessary for the carbohydrate sequences on these structures to represent the natural host cell receptors for EPEC. Although speculative, it is possible that the down-regulation of BfpA and intimin caused by soluble glycoconjugates, alone, might reduce the ability of EPEC to bind to their relevant receptors *in vivo* and this could form the basis for a novel therapeutic intervention in this gastrointestinal disease.

Another area for further investigation which arises from our results relates to the bacterial structure(s) the glycoconjugate(s) interact with to produce their effects. Thus far, only a role for BFP in EPEC LA has been well documented (9, 16, 18, 46-48). It is possible that the glycoconjugate(s) used in our experiments inhibited EPEC binding to HEp-2 cells by interfering with BFP mediated interactions. This inhibition could result from the glycoconjugate(s) interfering with the formation of microcolonies, as well as by blocking EPEC attachment to the surface of HEp-2 cells. It should be noted that, if this represents their mode of action, the glycoconjugate(s) would not, as proposed by Hicks *et al.*, be expected to prevent EPEC binding to intestinal tissue. However, in this situation, the total number of bacteria bound to these cells might still be reduced as a consequence of reduced microcolony formation. Overall, it remains to be determined whether BFP, or other unidentified adhesin(s), are involved in EPEC binding.

The inhibition results presented in this paper differ from those reported previously by Cravioto *et al.* (5). In their experiments, fucosylated pentasaccharides and difucosyllactose isolated from human milk were more effective inhibitors of EPEC binding to epithelial cells than their non-fucosylated backbone structures. Significantly, in their studies, fucosyllactose trisaccharides and lactose were not inhibitory. Based on these observations, it was suggested that the size of the oligosaccharide structure(s) might play a role in inhibition. In contrast to the results of Cravioto *et al.*, we found that the acetamido forms of LacNAc and LeX, conjugated to BSA, were effective inhibitors. Many factors might be responsible for these differing results. First, it is possible that EPEC, as a group, recognize more than one eukaryotic glycan structure. This could be related to serotype, geographical location or the number of times an organism was passaged in the laboratory (45). Alternatively, these differences could be due to the nature and presentation of the carbohydrate structures used to inhibit binding. In this respect, it is possible that the different conformations of the glycoconjugates (55), or the multivalency of these structures which, because of the multi-point interactions between microbes and eukaryotic cells in general, have been shown to be more effective inhibitors (24, 45), are responsible for these differences. Our laboratory is currently performing experiments with different EPEC serotypes, as well as with different fucosylated lactosyl structures as inhibitors, in order to investigate the roles of these factors in EPEC binding specificity. Regardless of the specific carbohydrate structures involved, our results provide a direction for further investigation of the regulation of EPEC attachment factors and whether similar processes are invoked upon bacterial binding to epithelial cells.

Despite our lack of understanding of the specific mechanisms by which EPEC binding to HEp-2 cells is inhibited, our results demonstrate the potential of these or similar compounds as therapeutic agents for treating EPEC infection. Our optimism is supported by results of other research groups which demonstrated that carbohydrates were able to reduce gastrointestinal bacterial load(s) *in vivo* (37, 45). In the past, it has been shown that EPEC mutants for BFP or intimin are still able to cause diarrhea in volunteers albeit at reduced levels (11, 35). By targeting more than one virulence factor, hopefully EPEC infections can be prevented. Since other human bacterial pathogens, for example, enterohemorrhagic *E. coli* and *Hafnia alvei*, cause attaching and effacing lesions similar to those observed in EPEC infections, (1, 13, 49), perhaps these bacteria could be future targets for a similar therapeutic strategy.

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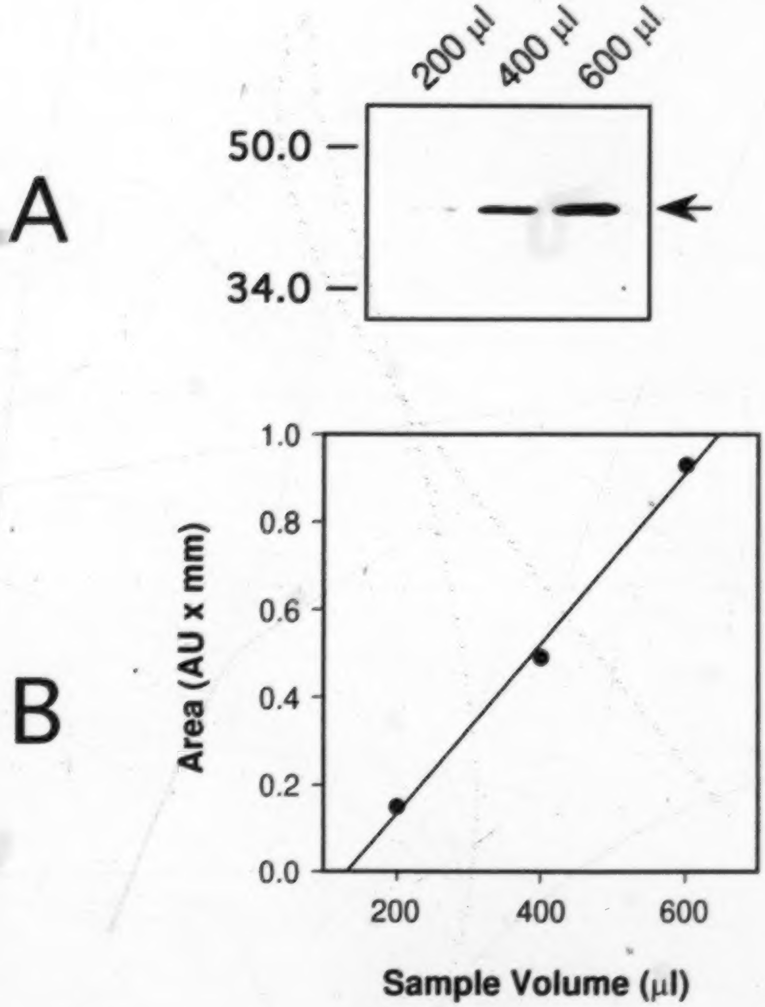
3.6. APPENDIX A

Figures indicated as "data not shown" in the manuscript, and additional data.

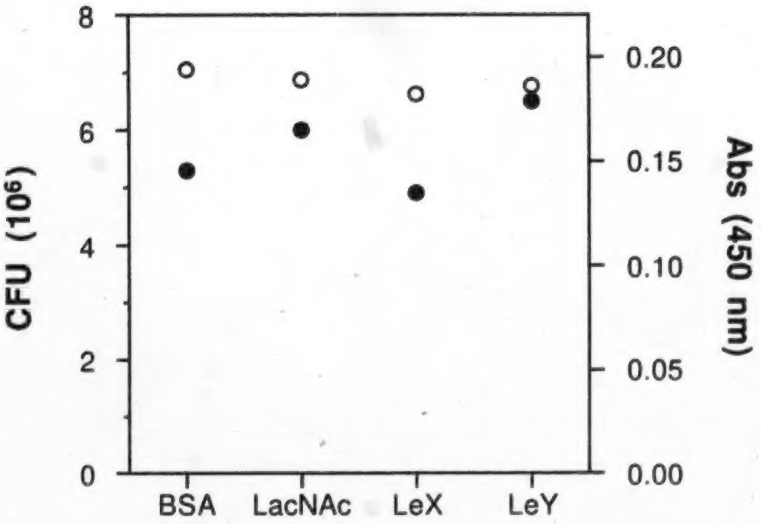
Figure 3.6. Linearity of the ECL response. EPEC E2348/69 was grown in DMEM for 1 h as described earlier. Next, 2, 4 or 6 x the volumes of bacterial culture, DMEM and BSA solution used in the binding inhibition experiments (final volume of 100 µl) were added to wells of a 48-well tissue culture plate. After incubating the plate for an additional 1 h in a CO_2 incubator, the bacteria were washed with PBS and separated by SDS-PAGE (12.5% polyacrylamide). Maltose-binding protein in each sample was then detected using the ECL detection system as described in Materials and Methods (A) and the levels were analyzed by densitometry. Plotting of the data indicated that the relationship between the amount of maltose-binding protein in each sample and the corresponding area determined by densitometry to be linear (B).



Linear Equation: $y = 0.002x - 0.259$
Correlation Coefficient: $r = 0.997$

Figure 3.7. Viabililty of EPEC after incubation with BSA-glycoconjugates. EPEC E2348/69 was cultured in DMEM for 1 h in a CO_2 incubator as described in Materials and Methods. Next, 32 ul of this culture, 56 µl of DMEM and 12 µl BSA-glycoconjugate were added to empty wells of a 96-well tissue culture plate. After incubating the plate in a CO_2 incubator for an additional 1 h, bacterial viability was determined using two methods. With the first method, the contents of each well were serially diluted in PBS and then plated onto TSA plates to determine the number of colony forming units (CFUs) in each sample (●).

Alternatively, since the number of CFU might be altered as a result of a glycoconjugate(s) inhibiting microcolony formation or causing the bacteria to aggregate, EPEC viability was also assessed using a modified XTT (sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay (**Stevens, M.G. and S.C. Olsen.** 1993. Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. J. Immun. Meth. **157**:225-231). This assay is based on the ability of viable bacteria to reduce XTT to soluble, orange formazan. Color development, which can be measured at 450 nm, is proportional to the number of viable bacteria. For this method, after DMEM-grown bacteria were incubated with the glycoconjugates for 1 h, the contents of each well were transferred into Luria broth (1:50 dilution). These cultures were incubated for 4 h at 37°C, after which time 400 µl of each culture and 100 µl of a solution consisting of XTT (1 mg/ml) and Coenzyme Q (25 µg/ml) were transferred to wells of a 48-well plate. After incubating the plate at 37°C for an additional 45 min, the cultures were transferred to microcentrifuge tubes and the bacteria were pelleted by centrifugation. The supernatant of each sample was collected and the Abs_{450} for each was recorded (○).


CFU (10^6)
Abs (450 nm)
8
6
4
2
0
0.20
0.15
0.10
0.05
0.00
BSA
LacNAc
LeX
LeY

CHAPTER 4

Effect of Carbon Source on Enteropathogenic *Escherichia coli* Localized Adherence

4. PREFACE

My investigation into a possible role for carbon source in enteropathogenic *Escherichia coli* (EPEC) adherence was prompted by the observations of two different research groups. In studies by Vuopio-Varkila and Schoolnik, it was found that the localized adherence (LA) phenotype of EPEC was promoted when bacteria were grown in tissue culture medium (5). This suggested that a component(s) of the medium was responsible for this effect. The second observation was from work by Nishikawa *et al.* which demonstrated that the attachment of some enterohemorrhagic *E. coli* (EHEC) strains to epithelial cells was reduced when bacteria were grown in media supplemented with carbohydrates that were metabolized by the bacteria (4). The decrease in EHEC binding, in particular with glucose, was attributed to catabolite repression. These results appeared to contrast what had been demonstrated for EPEC, that is, LA was induced by growing bacteria in tissue culture media (5), which usually includes glucose [Gibco product catalog]. Since EHEC and EPEC express homologous intimins which are involved in the formation of attaching and effacing lesions (1-3), these results were intriguing because they suggested that EHEC and EPEC binding might be regulated differently. Based on the observations of both groups, I performed experiments to determine the effect of carbon source on EPEC attachment. In contrast to the adherence observed when EPEC were grown in tissue culture medium containing glucose, I found that bacteria grown in the presence of galactose did not adhere to epithelial cells despite similar numbers of colony forming units being incubated with the monolayers. At this point, instead of further examining the differences between EPEC and EHEC binding, my research focused on the effects of glucose and galactose on EPEC binding.

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4.1. INTRODUCTION*

Historically, the term enteropathogenic *Escherichia coli* (EPEC) referred to *E. coli* strains of specific serotypes which caused diarrhea in infants (28, 35). The enterovirulent *E. coli* are now divided into subgroups according to unique virulence mechanisms (8). Clinical symptoms of EPEC infection result from bacterial colonization of the intestine (39, 47, 51) and the subsequent formation of attaching and effacing (AE) lesions (33). Initially, EPEC bind to epithelial cells as discrete clusters of bacteria which are localized at a few sites on the cell surface (4, 41). *In vitro*, bacteria grown under conditions which promote attachment also secrete several proteins (including the *espA* (25) and *eaeB* (*espB*) gene products (9, 13, 25)) which are associated with virulence (20, 21, 24). Following initial attachment, EPEC activate signal transduction processes involving protein phosphorylation (2, 30, 36) and increased levels of intracellular calcium (3, 11) and inositol phosphates (11, 13, 14) in epithelial cells. The intracellular messengers generated, in turn, cause cytoskeletal rearrangement which results in the effacement of enterocyte microvilli and the accumulation of cytoskeletal components at sites of bacterial attachment (12, 26, 30). Expression of the EPEC *eaeA* gene product (intimin) (19, 22, 23) is required for the organization of cytoskeletal components beneath the bacteria and the subsequent formation of a pedestal-like structure with which the bacteria become intimately associated (26, 33, 36). Recently, intimin was shown to bind to a 90-kDa host tyrosine-phosphorylated protein which accumulates at sites of bacterial attachment (38). McDaniel *et al.* identified a genetic region, referred to as the locus of enterocyte effacement, in which the genes thus far associated with the AE activity of EPEC are located (32).

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Although processes involved in the formation of the AE lesions have been elucidated, less is known about the initial binding of EPEC to epithelial cells. Cravioto *et al.* demonstrated that EPEC attachment to epithelial cells was mannose resistant, suggesting that type I pili were not responsible for the localized-adherence (LA) phenotype (4). Subsequently, EPEC were found to possess a high-molecular-weight EPEC adherence factor (EAF) plasmid which is associated with binding (1). Although several reports described an adhesin(s) which may play a role in attachment (18, 40, 42, 50), the observation that EPEC LA is induced by specific growth conditions helped to identify the factor(s) which mediates binding.

Giron *et al.* described unique rope-like structures, called bundle-forming pili (BFP), which promoted the formation of microcolonies when the bacteria were grown on blood agar plates or while attached to epithelial cells (17). A role for BFP in LA was supported by results demonstrating that bacteria lacking the EAF plasmid did not produce BFP and that antibodies raised to BFP partially inhibited EPEC binding. Similarly, Vuopio-Varkila and Schoolnik found that EPEC microcolony formation and adherence were induced by transferring bacteria from a relatively undefined bacterial growth medium (Luria Broth [LB]) to a defined tissue culture medium (DME with or without fetal bovine serum [FBS]) (49). In those experiments, induction of LA coincided with increased expression of several bacterial outer membrane proteins, including an 18.5-kDa protein which was homologous to the BFP subunit. More recent studies demonstrated that EPEC mutants with Tn*phoA* insertions in the gene encoding the BFP structural subunit (*bfpA*) no longer expressed the LA phenotype (7, 43). Although reintroduction of the *bfpA* gene into these mutants restored BFP expression, the bacteria were unable to adhere in a LA pattern, suggesting that other genes were required for attachment. Recently, the *bfpA* gene was shown to be part of a gene cluster involved in BFP biosynthesis and LA (44, 45).

Since the LA phenotype is expressed when EPEC are transferred to tissue culture medium (49), we investigated whether specific components of the medium were involved in mediating bacterial attachment. In this report, we describe the effect of carbon source on EPEC binding. Our results provide a model system to further investigate the role of carbon source in the regulation of EPEC adherence and virulence.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains. Bacterial strains E2348/69, JPN15, *cfm* 27-3-2(1) and *eaeA* 10-5-1(1) were kindly provided by B.B. Finlay (University of British Columbia, Vancouver, British Columbia, Canada). EPEC E2348/69 (O127:H6) is a wild-type strain isolated from an infant with gastroenteritis (29). JPN15 is a derivative of E2348/69 which lacks the EAF plasmid associated with the LA phenotype (23). Strains *cfm* 27-3-2(1) (5, 21, 32, 36) and *eaeA* 10-5-1(1) (5, 22, 23, 32, 36) are Tn*phoA* mutants of E2348/69 which have mutations in the *sepA* and *eaeA* genes, respectively. The strain *bfpA* 31-6-1(1), generously provided by M.S. Donnenberg (University of Maryland School of Medicine, Baltimore, Md.), is also derived from E2348/69 and has a Tn*phoA* insertion in the *bfpA* gene (5, 7). EPEC O111:H2 and O119:H6 are wild-type strains which have been described previously (48). Dulbecco's modified Eagle medium (DMEM) (catalog no. 23800; Gibco, Burlington, Ontario, Canada) was supplemented with 44 mM NaHCO3, 40 μM phenol red and 25 mM (0.45%) glucose or galactose. Throughout all experiments, including coincubations with tissue culture cells, the bacteria were cultured in DMEM without FBS.

All bacterial strains were incubated overnight at 37°C in tryptic soy broth (Difco, Detroit, Mich.) under normal atmospheric conditions. The cultures were then inoculated (1:100) into carbohydrate-supplemented DMEM (preequilibrated to tissue culture conditions by incubation overnight in a humidified atmosphere of 5% CO_2-95% air) and incubated for various periods of time in a CO_2 incubator until their use in experiments.

4.2.2. Tissue culture. HEp-2 cells (CCL-23) were obtained from the American Type Culture Collection (Rockville, Md.). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2-95% air in minimal essential medium supplemented with 10% FBS (Gibco). Subconfluent monolayers were prepared by disrupting HEp-2 cells with a solution consisting of 0.25% (vol/vol) trypsin in FC buffer (0.14 M NaCl, 5.0 mM KCl, 20.0 mM Tris-HCl, 5.0 mM Tris base, 0.5 mM EDTA [pH 7.2]). The cells were resuspended in tissue culture medium and approximately 1 x 10^5 cells were seeded onto 12-mm-diameter glass coverslips in 24-well tissue culture plates. The plates were then incubated at 37 °C for use in experiments the following day.

4.2.3. EPEC LA to tissue culture cells. EPEC strains were grown in glucose- or galactose-supplemented DMEM (in the absence of HEp-2 cells) as described above. Subconfluent monolayers of HEp-2 cells were prepared in 24-well tissue culture plates as described above. After the monolayers were washed twice with phosphate-buffered saline (PBS), 900 µl of log-phase bacterial culture (5 x 10^7 - 5 x 10^8 CFU grown in DMEM) was added to each well. Depending on the experiment, bacterial cultures were either added directly to the HEp-2 cells or were washed three times and resuspended in fresh preequilibrated DMEM (without FBS and with 25 mM glucose or galactose or no carbon source) immediately before their addition to the tissue culture wells. The latter experiments were performed to determine if wild-type bacteria had to be grown in the presence of a particular carbon source to express the attachment phenotype or if the carbohydrates directly affected bacterial binding. Unless otherwise indicated, mannose (1% wt/vol) was included during the incubation of bacteria with the HEp-2 cells to inhibit type I mediated bacterial attachment (4). Following incubation of the bacteria with the HEp-2 cells at 37°C for 30 min, the monolayers were washed five times with PBS and then fixed for 10 min with methanol. This relatively short coincubation period was chosen to allow bacterial binding to occur while minimizing a possible influence of HEp-2 cells on the expression of an attachment factor(s) by EPEC. Bacterial adherence was determined by staining the cells with Giemsa stain and viewing the coverslips with the 100x objective lens of a Reichert light microscope. A minimum of 100 randomly chosen HEp-2 cells were observed. HEp-2 cells which had attached microcolonies consisting of more than 4 bacteria were considered positive for having EPEC with the LA phenotype (49). Experiments were performed three times, each time in duplicate.

4.2.4. Biotinylation of bacterial proteins. Biotinylations were performed by a modified surface-labeling procedure (Pierce, Rockford, Ill.) as described by the manufacturer. Mid-log-phase bacteria were grown in glucose- or galactose-supplemented DMEM (in the absence of HEp-2 cells) as described above. For experiments involving EPEC mutant *cfm* 27-3-2(1), *eaeA* 10-5-1(1), or *bfpA* 31-6-1(1), the bacteria were incubated in tissue culture medium for periods of time (i.e., 3 h) equivalent to those for the wild-type strain E2348/69. The bacteria (approximately 3 x 10^9 cells) were washed twice with PBS (pH 8.0) and resuspended in 1 ml PBS (pH 8.0). The samples were transferred to microcentrifuge tubes and 15 µl of biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester (20 mg/ml in H_2O) (Sigma, St. Louis, Mo.) was added to each tube. After incubation on an end-over-end rotator for 1 h at room temperature, the cells were washed by centrifugation four times with PBS (pH 6.0) and once with PBS (pH 7.4). Bacterial whole-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) in the presence of 50 mM dithiothreitol and then transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, Mass.).

4.2.5. Detection of biotinylated proteins. After transfer of the bacterial proteins to membranes, the nonspecific binding sites were blocked by incubating the membranes in a solution consisting of 5% (wt/vol) skim milk in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 0.05% Tween-20 (TBST) for 2 h at room temperature. The membranes were incubated for an additional 2 h at room temperature with a streptavidin-alkaline phosphatase conjugate (Sigma) in 5% skim milk-TBST and then washed three times with TBST. Biotinylated proteins were visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) color development reagents.

4.2.6. Immunoblotting of bacterial proteins. Polyclonal rabbit anti-intimin (22) and anti-BfpA (52) antibodies were generously provided by J.B. Kaper and M.S. Donnenberg (University of Maryland School of Medicine, Baltimore), respectively. Bacterial whole-cell lysates were transferred to Immobilon-P membrane as described above. After being blocked with 5% skim milk-TBST, the membranes were incubated with anti-intimin (1:2,000) or anti-BfpA (1:4,000) antibodies in 5% skim milk-TBST for 2 h at room temperature. The membranes were washed three times with TBST and then incubated with goat anti-rabbit immunoglobulin G antibodies conjugated to alkaline phosphatase in 5% skim milk-TBST. The membranes were washed again with TBST, and proteins were detected with NBT and BCIP color development reagents.

4.2.7. Statistical analysis. The significance of any differences in bacterial binding was determined by Student's *t* test.

4.3. RESULTS

4.3.1. Bacterial adherence to HEp-2 cells. Previous results suggested that a component of tissue culture medium was involved in regulating EPEC LA to epithelial cells (49). Preliminary experiments performed in our laboratory revealed that wild-type EPEC grown in DMEM with glucose, fructose, or mannose adhered to HEp-2 cells in an LA pattern, whereas bacteria grown in the presence of galactose did not (data not shown; [Appendix A, Figure 4.4.]). Based on these results, we selected glucose and galactose as carbon sources to further investigate their role in bacterial attachment. Glucose was chosen (instead of fructose or mannose) to promote the adherence phenotype since most experiments involving EPEC binding to epithelial cells are performed with tissue culture medium supplemented with glucose.

Growth curves were initially prepared for wild-type EPEC isolate E2348/69 (O127:H6) (wild-type) and JPN15 (a mutant of E2348/69 lacking the EAF plasmid) cultured in glucose- or galactose-supplemented DMEM to confirm that the bacteria were in log-phase growth [Appendix A, Figure 4.5.], since it was shown previously that EPEC attached to epithelial cells during this stage of growth (49). The ability of the bacteria to utilize glucose or galactose as a carbon source was indicated by a decrease in the pH of the culture media over time (data not shown). Incubation periods used to grow the bacteria in DMEM (before addition to the tissue culture wells) were selected based on our observation that wild-type E2348/69 grown in DMEM containing glucose attached in significant numbers to HEp-2 cells during early- to mid-log-phase growth. Overall, bacterial binding was examined during the early log to mid-log, mid-log, and late log phases of growth to ensure that any differences in bacterial attachment were not due to differences in the stage of log-phase growth of the cultures. The results of these experiments demonstrated that only E2348/69 grown in glucose-supplemented medium attached to HEp-2 cells (Table 4.1.). Early- to mid-log-phase wild-type bacteria grown in DMEM with glucose attached to 82% of the HEp-2 cells, while bacteria in later stages of log-phase growth attached to approximately 96% of the HEp-2 cells.

In contrast, E2348/69 incubated in DMEM containing galactose did not adhere to HEp-2 cells at any stage of log-phase growth. This result was similar to that seen when JPN15, which also did not bind to HEp-2 cells, was used in these experiments. Although previous reports demonstrated that EPEC lacking the EAF plasmid (i.e., JPN15) is able to adhere to epithelial cells, albeit at reduced levels and requiring prolonged incubation periods of the bacteria with tissue culture cells (23, 27), no adherence was observed in our assays, presumably because of the relatively short period of coincubation (i.e., 30 min) of the bacteria with the HEp-2 monolayers. The observed differences in bacterial attachment were not due to differences in the number of CFU added to the tissue culture wells or to differences in the pH of the cultures, since these were similar for the various cultures at each stage of log-phase growth (data not shown; [Appendix A, Figure 4.6.]).

To determine whether the effect of carbon source on EPEC attachment was unique to isolate E2348/69, we repeated these experiments with wild-type EPEC of different serotypes. Using EPEC isolates O111:H2 and O119:H6 which have been described previously (48), we found that these strains also adhered to HEp-2 cells when the bacteria

Table 4.1. EPEC Adherence to HEp-2 Cells

E. coli Strain	Addition to DMEM	Growth Phase (log)	Incubation Period (h)[a]	% HEp-2 Cells with LA EPEC[b]
E2348/69 (wild-type)	Glucose	Early to Mid	2	82.7 ± 3.7
		Mid	3	96.1 ± 0.2
		Late	4	96.0 ± 0.8
	Galactose	Early to Mid	6	0
		Mid	10.5	0
		Late	16	0
JPN15	Glucose	Early to Mid	2.5	0
		Mid	3.5	0
		Late	4.5	0

[a] Time of incubation of bacteria in DMEM before their addition to the tissue culture wells.

[b] Means ± standard deviations from three independent experiments.

were grown in DMEM containing glucose but not when the bacteria were grown in DMEM supplemented with galactose (Table 4.2. [and Appendix A, Figure 4.7.]).

4.3.2. Role of carbon source in bacterial attachment. Additional experiments were performed to determine if it was necessary for wild-type EPEC to be grown in the presence of a specific carbon source in order to express a particular attachment phenotype or if the carbohydrates had a direct function in promoting (glucose) or inhibiting (galactose) bacterial attachment. For this, wild-type EPEC E2348/69 was grown in glucose- or galactose-supplemented DMEM to mid-log-phase. The bacteria were then washed and resuspended in fresh DMEM containing either glucose, galactose, or no carbon source immediately before being incubated with the HEp-2 monolayers. We found no significant difference (*P*=.07) in the binding ability of E2348/69 initially incubated in DMEM supplemented with glucose, whether the bacteria were washed and resuspended in medium alone or in medium containing glucose or galactose prior to their coincubation with the HEp-2 cells (Table 4.3.). On the other hand, E2348/69 grown in DMEM containing galactose did not adhere to HEp-2 cells regardless of the medium used to wash and resuspend the bacteria.

Similar binding results were obtained when the adherence assays were performed in the absence of mannose (Table 4.3.). This indicated that the pattern of bacterial attachment observed in earlier experiments was not due to the inclusion of mannose during the period of coincubation of bacteria with the HEp-2 cells. Overall, these results suggest that it is the growth of EPEC in the presence of a specific carbon source which influences its attachment to HEp-2 cells, since incubation of the bacteria in the presence of a different sugar (or no sugar) during the 30-min coincubation with HEp-2 cells did not significantly alter their binding to epithelial cells.

Table 4.2. Adherence of Wild-type EPEC of Different Serotypes to HEp-2 Cells

EPEC Serotype	Addition to DMEM	Incubation Period (h)[a]	% HEp-2 Cells with LA EPEC[b]
O111:H2	Glucose	3	86.4 ± 3.4
	Galactose	8.5	0
O119:H6	Glucose	3	57.0 ± 10.7
	Galactose	8.5	0

[a] Incubation of bacteria in DMEM to approximately mid-log-phase before addition to the tissue culture wells [Appendix A, Figure 4.7.].

[b] Mean ± standard deviations from three independent experiments.

Table 4.3. Effect of Medium Washes on Wild-type EPEC E2348/69 Adherence to HEp-2 Cells

Addition to DMEM for:		% HEp-2 Cells with LA EPEC[c]	
Growth[a]	Washing and Resuspension[b]	with D-mannose	without D-mannose
Glucose	Glucose	86.5 ± 4.1	92.4 ± 7.7
	Galactose	81.8 ± 3.7	83.4 ± 9.1
	None	86.3 ± 4.2	90.3 ± 5.6
Galactose	Glucose	0	0
	Galactose	0	0
	None	0	0

[a] Refers to the incubation of bacteria in carbohydrate-supplemented DMEM before washing and incubating with HEp-2 cells.

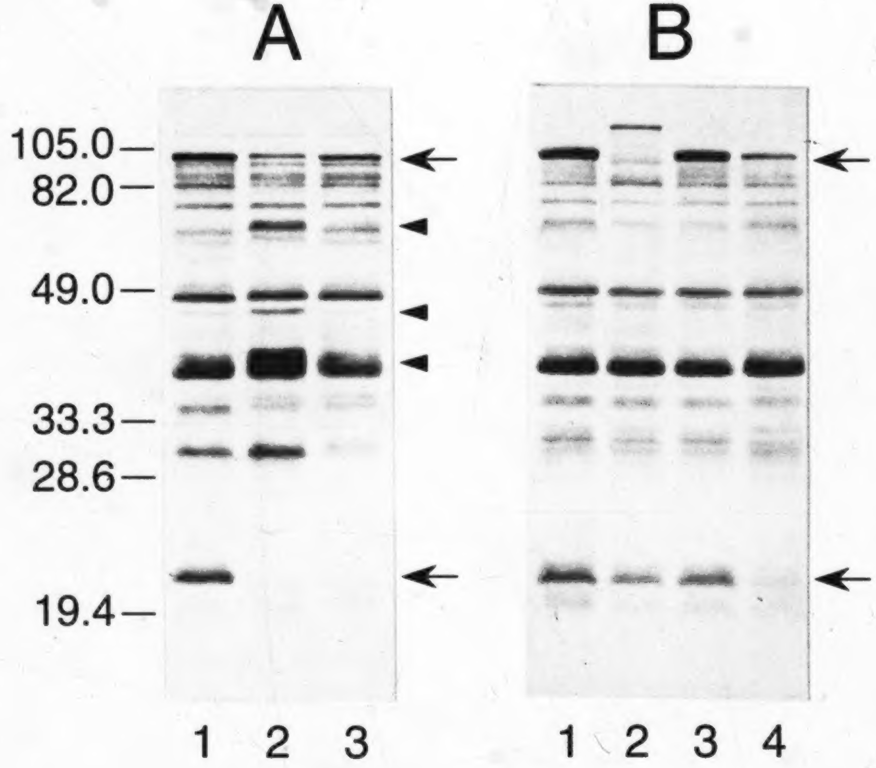
[b] Media used to wash and resuspend mid-log phase bacteria immediately before their incubation with HEp-2 cells.

[c] Means ± standard deviations of three independent experiments.

4.3.3. Proteins expressed by bacteria grown in media with different carbon sources. Since EPEC LA is associated with the increased expression of several proteins (17, 49), we investigated whether the different attachment phenotypes observed in our experiments also correlated with the expression of specific proteins. Using a surface biotinylation procedure to label bacterial proteins, we observed an increased expression of 94- and 22-kDa proteins in the lysates of adherent E2348/69 grown in DMEM containing glucose compared to nonadherent E2348/69 or JPN15 grown in medium containing galactose or glucose, respectively (Figure 4.1.A). Although the nonadherent cultures expressed lower levels of a 94-kDa protein, these levels were visible in all experiments. In contrast, a 22-kDa protein was observed in the lysates of nonadherent strains in only some of our experiments and at levels which were difficult to detect. Wild-type E2348/69 grown in medium with galactose expressed increased levels of 38- to 41-, 46- and 68-kDa proteins, which were not expressed when the wild-type strain or its derivative JPN15, lacking the EAF plasmid, was grown in medium containing glucose.

4.3.4. Proteins expressed by EPEC mutants grown in medium containing glucose. Mutants of EPEC isolate E2348/69 which are defective at various stages of attachment to epithelial cells were also biotinylated, and the protein profiles were compared to those of the wild-type strain grown under conditions which induced LA. Both the *cfm* 27-3-2(1) and *eaeA* 10-5-1(1) mutants have been characterized previously. The *cfm* 27-3-2(1) mutant (5, 21, 32, 36) has two chromosomal Tn*phoA* insertions. One insertion occurs in the *sepA* gene of the recently described type III secretory pathway of EPEC. Phenotypically, these bacteria do not initiate signal transduction processes in epithelial cells and have greatly reduced fluorescent actin staining activity (which correlates with AE lesion formation (26)). However, these mutants are still able to attach to epithelial cells in a

Figure 4.1. Analysis of biotinylated EPEC proteins. Mid-log-phase DMEM cultures of wild-type EPEC E2348/69 or mutants of this strain were grown in carbohydrate-supplemented DMEM as described in the text. The bacteria were labeled with biotin, washed to remove unincorporated label, and digested in sample buffer. After separation by SDS-PAGE (12.5% polyacrylamide), the biotinylated proteins were detected by using streptavidin conjugated to alkaline phosphatase. Equivalent amounts of protein were loaded per lane. (A) Lanes: 1, E2348/69 with glucose; 2, E2348/69 with galactose; 3, JPN15 with glucose. (B) Lanes: 1, E2348/69 with glucose; 2, *eaeA* 10-5-1(1) with glucose; 3, *cfm* 27-3-2(1) with glucose; 4, JPN15 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are indicated on the left. Proteins expressed at higher levels by E2348/69 with glucose (arrows) and E2348/69 with galactose (triangles) are indicated.


A
B
105.0
82.0
49.0
33.3
28.6
19.4
1
2
3
1
2
3
4

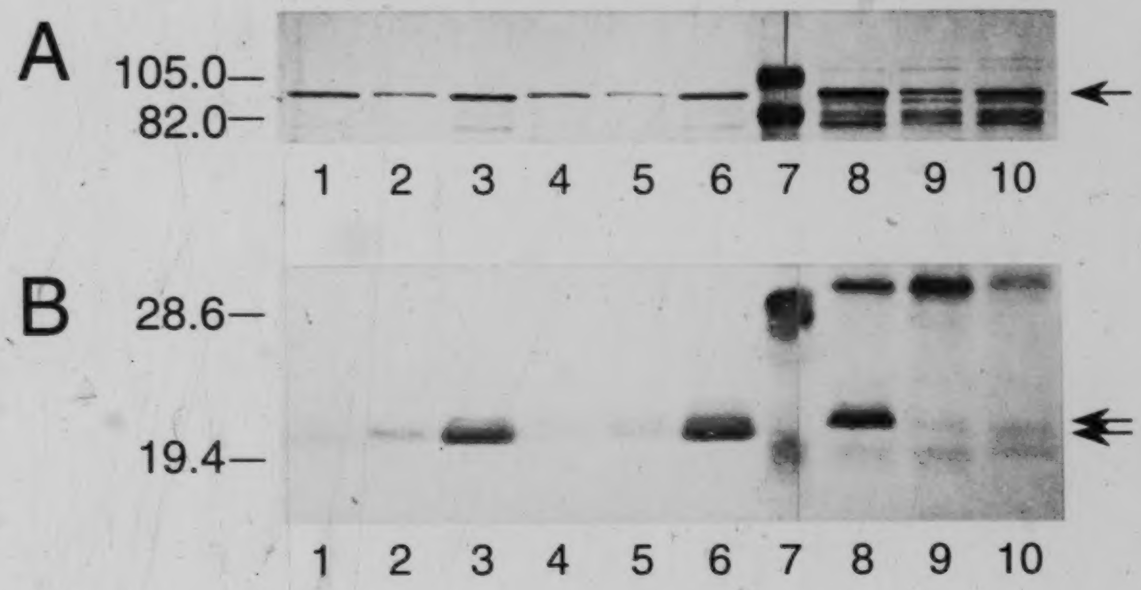
localized pattern. The *eaeA* 10-5-1(1) mutant (5, 22, 23, 32, 36), on the other hand, has a single Tn*phoA* insertion in the *eaeA* gene which codes for intimin. Although this mutant is able to attach to epithelial cells in a LA pattern and to generate intracellular messengers in host cells, it is unable to mediate AE lesion formation.

A 94-kDa protein was expressed at higher levels by the LA-positive strains, with the exception of the *eaeA*::Tn*phoA* mutant. This mutant expressed a protein (molecular size of more than 105 kDa) which was not observed in the lysates of the other bacterial strains. Also, there was increased expression of a biotinylated 22-kDa protein in the lysates of all strains which demonstrate the LA phenotype (i.e., E2348/69, and *cfm* 27-3-2(1) and *eaeA* 10-5-1(1) mutants) (Figure 4.1.B). A protein with a similar molecular mass was also expressed by the nonadherent (EAF plasmidless) JPN15 strain but at levels much lower than those in adherent bacteria (Figure 4.1.B).

4.3.5. Effect of carbon source on intimin and BfpA expression.

Immunoblotting was performed with wild-type EPEC E2348/69 and JPN15 to determine if the adherence phenotype was associated with the expression of proteins shown previously to be associated with EPEC attachment (15, 17, 38, 49). Overall, the mobilities of proteins detected with anti-intimin or anti-BfpA antibodies were similar regardless of whether the bacteria were biotinylated or not (Figure 4.2., lanes 1 to 6). The anti-intimin serum recognized a 94-kDa protein which was expressed at higher levels by adherent bacteria (E2348/69 with glucose) than by nonadherent bacteria (E2348/69 with galactose and JPN15 with glucose). In addition to having the same mobility as intimin, the levels of expression of the biotinylated 94-kDa protein corresponded to the levels of intimin production by the same strains.

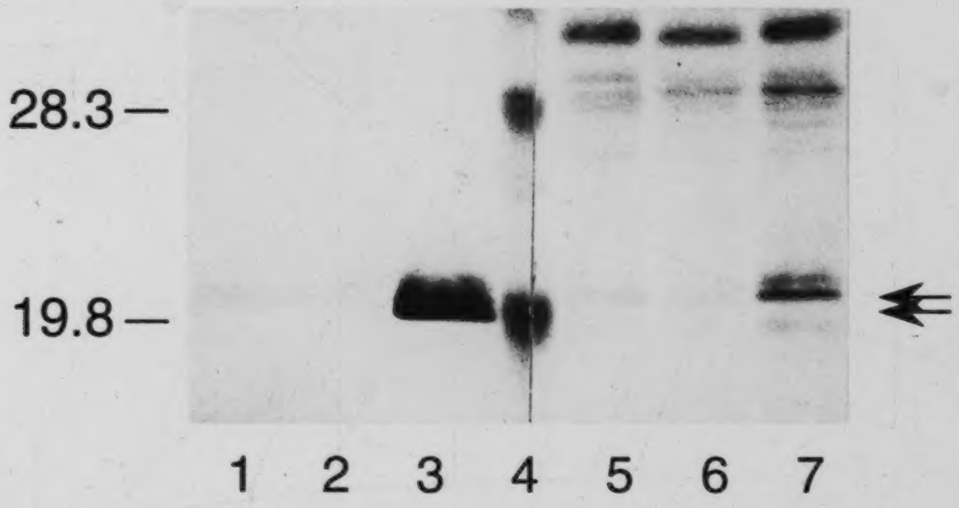
Figure 4.2. Alignment of immunoblots with biotinylated proteins of wild-type EPEC E2348/69 and JPN15. Whole-cell lysates of unbiotinylated or biotinylated bacteria were separated by SDS-PAGE (12.5% polyacrylamide) and transferred to Immobilon-P membranes. Proteins were detected with anti-intimin (A) (lanes 1 to 6) or anti-BfpA (B) (lanes 1 to 6) antibodies, and their mobilities were compared to those of biotinylated proteins (lanes 8 to 10). The order of samples loaded in lanes 1 to 3 and lanes 4 to 6 is opposite to the order of samples loaded in lanes 8 to 10 to facilitate comparison of the molecular sizes of proteins. The relative mobilities of the bacterial proteins after realignment of the membranes according to the mobilities of prestained molecular size standards are shown. Lanes: 1, unbiotinylated JPN15 with glucose; 2, unbiotinylated E2348/69 with galactose; 3, unbiotinylated E2348/69 with glucose; 4, biotinylated JPN15 with glucose; 5, biotinylated E2348/69 with galactose; 6, biotinylated E2348/69 with glucose; 7, prestained molecular size standards; 8, biotinylated E2348/69 with glucose; 9, biotinylated E2348/69 with galactose; 10, biotinylated JPN15 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are shown on the left. Arrows indicate 94-kDa and 20- to 22-kDa proteins.


A
105.0—
82.0—
1 2 3 4 5 6 7 8 9 10
B
28.6—
19.4—
1 2 3 4 5 6 7 8 9 10

Using anti-BfpA antibodies, we observed increased expression of the BFP subunit in [illegible] lysates of wild-type E2348/69 grown under glucose-supplemented conditions which promoted attachment. Low levels of the BFP subunit were also detected in the lysates of nonadherent E2348/69 grown in DMEM containing galactose but not in the lysates of JPN15, which lacks the EAF plasmid. Although expression of both BfpA and a biotinylated 22-kDa protein was promoted by glucose, SDS-PAGE revealed a slight difference in the mobilities of these proteins. The molecular size of the protein recognized by BfpA antibodies was calculated to be 20 kDa.

4.3.6. Role for the 22-kDa biotinylated protein in adherence. The slight difference in the molecular sizes of a biotinylated 22-kDa protein and BfpA in some experiments led us to perform additional experiments using the mutant *bfpA* 31-6-1(1) to determine whether expression of the 22-kDa protein was associated with the LA phenotype. The *bfpA* 31-6-1(1) mutant has a single Tn*phoA* insertion in the *bfpA* gene which codes for the structural subunit of BFP (7). The requirement for additional sequences downstream of the *bfpA* gene to restore the LA phenotype in these mutants, however, suggests that other genes in addition to the *bfpA* gene are affected by this mutation. This mutant was shown previously to be incapable of adhering to epithelial cells in a LA pattern (5). Our results demonstrate that the level of a biotinylated 22-kDa protein expressed by the *bfpA* 31-6-1(1) mutant was lower than that expressed by wild-type E2348/69 and similar to the level observed with the LA-negative strain JPN15 (1, 23) (Figure 4.3.).

Figure 4.3. Association of a 22-kDa biotinylated protein with the LA phenotype. Log-phase bacteria grown in DMEM containing glucose were biotinylated and the cell lysates were separated by SDS-PAGE (12.5% polyacrylamide). After transfer to polyvinylidene difluoride membranes, the biotinylated proteins were detected with either anti-BfpA antibodies (lanes 1 to 3) or streptavidin conjugated to alkaline phosphatase (lanes 5 to 7). Equivalent amounts of protein were loaded per lane. Lanes: 1, JPN15 with glucose; 2, *bfpA* 31-6-1(1) with glucose; 3, E2348/69 with glucose; 4, molecular size standards; 5, JPN15 with glucose; 6, *bfpA* 31-6-1(1) with glucose; 7, E2348/69 with glucose. The mobilities of the prestained molecular size standards (in kilodaltons) are indicated on the left. BfpA and a 22-kDa biotin-labeled protein are indicated by arrows.



28.3 —
19.8 —
1
2
3
4
5
6
7

4.4. DISCUSSION

The expression of virulence factors by many pathogenic bacteria is environmentally regulated (10, 31, 46). EPEC LA, which is considered to be important for pathogenesis, is an inducible phenotype mediated by specific growth conditions. Previous reports demonstrated that EPEC growth on blood agar plates (17), in tissue culture medium (7, 49), or in the presence of calcium or ammonium (34) regulates the production of BFP or expression of the LA phenotype. Temperature also affects BFP expression (7, 34). In this report, we demonstrate that the carbon source also plays a role in EPEC attachment. Wild-type EPEC grown in DMEM containing glucose adhered to HEp-2 cells, while the attachment of bacteria grown in medium supplemented with galactose was reduced to levels obtained with JPN15, a strain lacking the EAF plasmid which is essentially nonadherent. Expression of the attachment phenotype required the bacteria to be grown in medium containing a specific carbon source, since changing the culture medium prior to incubating the bacteria with the HEp-2 cells did not alter bacterial binding.

An additional factor associated with EPEC virulence is the ability of EPEC to invade epithelial cells (6). Recently, Geyid *et al.* demonstrated that invasion of wild-type EPEC E2348 (O127:H6) into Caco-2 cells was reduced when the bacteria were grown in LB containing 1% glucose compared to bacteria grown in LB alone (16). In their experiments, however, invasion was examined independently from bacterial attachment, since the bacteria were centrifuged onto the epithelial cell monolayers to initiate the invasion process. In view of these results, we repeated our binding assays with DMEM supplemented with 1% glucose to determine if an increased level of glucose in the medium would reduce EPEC attachment to HEp-2 cells. We did not observe a decrease in the LA of EPEC E2348/69 in these experiments compared to that in earlier experiments in which the bacteria were grown in DMEM with 0.45% glucose (data not shown; [Appendix A, Figure 4.8.]). Because of the differences in the protocols used to investigate attachment and invasion in these experiments, many factors could contribute to the different effects of glucose on EPEC virulence factors, including the presence of other components in the different bacterial growth media (i.e., LB versus DMEM) or the different epithelial cell lines used in the experiments. Alternatively, these results present the interesting possibility that EPEC binding and invasion may be affected differently by carbon source.

The LA phenotype of EPEC is associated with increased expression of several outer membrane proteins. The best characterized of these proteins are intimin, which is required for intimate attachment (22, 23, 36), and BfpA, which is associated with LA (17, 49). Intimin is a 94-kDa outer membrane protein required for the organization of host cytoskeletal components at sites of bacterial attachment and for the formation of AE lesions. Although intimin is chromosomally encoded, its expression is upregulated by the *per* (plasmid-encoded regulator) region of the EAF plasmid (19). BfpA, on the other hand, is encoded on the EAF plasmid and is associated with the initial binding of EPEC microcolonies to epithelial cells. BfpA is the major structural subunit of BFP and has a predicted molecular size of 18.7 kDa (7, 43).

Using wild-type EPEC strain E2348/69, we found that intimin and BfpA were expressed at higher levels by adherent bacteria than by nonadherent strains (17, 19, 49), which is similar to results reported previously. Furthermore, we observed that the levels of intimin and BfpA expressed by E2348/69 grown in galactose were similar to those observed with JPN15 which lacks the EAF plasmid. Since expression of BFP is required for the LA phenotype (7, 17, 43), these results suggest that the reduced ability of wild-type EPEC grown in DMEM with galactose to attach to HEp-2 cells might also result from lower levels of BfpA expression. Using biotinylation techniques to examine the expression of proteins associated with LA, we identified a 94-kDa protein as intimin based on the similarity of its mobility and relative level of expression by various cultures. Expression of a biotinylated 22-kDa protein was similar to that of BfpA in that it was expressed at higher levels by adherent bacteria than by nonadherent cultures. However, since a slight difference in the mobilities of these proteins was observed in most experiments (Figure 4.2.B), we are currently performing additional experiments to determine whether these proteins are distinct, and if so, the identity of the 22-kDa protein. Nevertheless, our results indicate that expression of the biotinylated 22-kDa protein is associated with the LA phenotype, since the level of expression of this protein by the *bfpA* 31-6-1(1) mutant was similar to that observed with JPN15.

In addition to the production of BFP (7, 17, 34, 49), protein secretion (20, 24), induction of host tyrosine phosphorylation (24), and AE lesion formation (37) by EPEC are also regulated by growth conditions. The specific mechanisms by which these factors affect the expression of virulence factors, however, has not yet been determined. Overall, these results suggest that the regulation of EPEC virulence factors is likely to be complex and that several environmental parameters may be involved. The observation that EPEC binding is affected by carbon source provides a model system which can be used to investigate the regulation of EPEC attachment. Since colonization of the intestinal mucosa is important for EPEC pathogenesis, a better understanding of the regulation of these processes will assist in the development of potential therapies for EPEC-mediated diarrhea.

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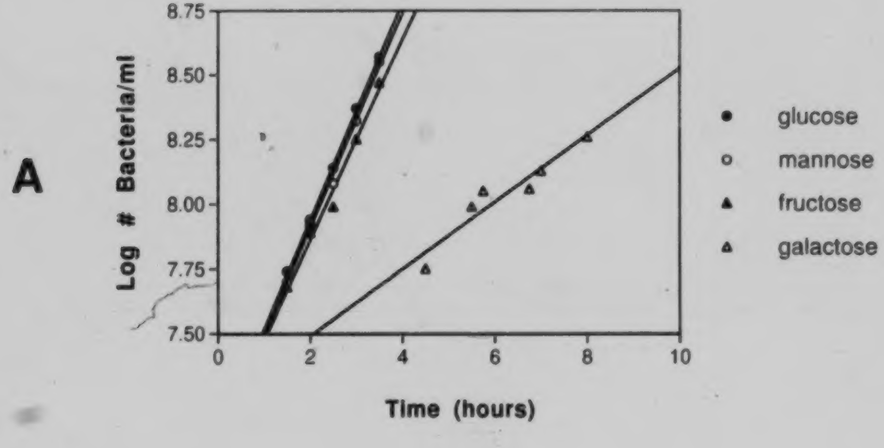
4.6 APPENDIX A

Figures indicated as "data not shown" in text, and additional figure.

Figure 4.4. Preliminary results for the effect(s) of carbon source on EPEC attachment. EPEC E2348/69 was cultured in DMEM supplemented with glucose, mannose, fructose, or galactose for 2.7, 2.8, 2.9 and 7.7 h, respectively, at 37°C in a CO_2 incubator. Approximately 1.7 x 10^8 bacteria, in a final volume of 1 ml (determined using the Petroff-Hausser counting chamber) (A), were then added to wells of a 24-well tissue culture plate containing sub-confluent HEp-2 cell monolayers grown on glass coverslips. After incubating for an additional 30 min in a CO_2 incubator, the cells were washed with PBS, fixed with methanol and stained with Giemsa stain. The number of HEp-2 cells with LA EPEC was determined as described in Materials and Methods of this chapter (B).


A
Log # Bacteria/ml
8.75
8.50
8.25
8.00
7.75
7.50
0
2
4
6
8
10
Time (hours)
● glucose
○ mannose
▲ fructose
△ galactose

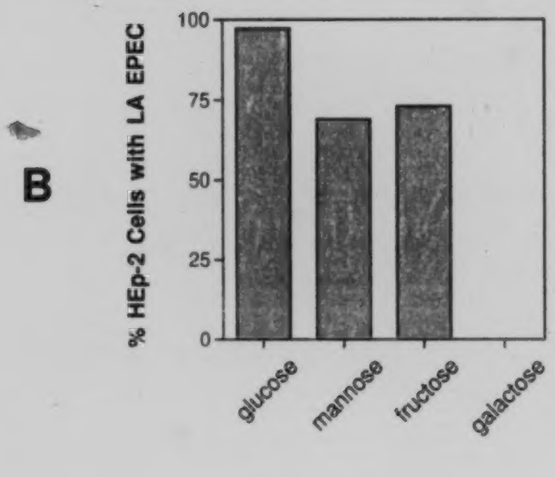

B
% HEp-2 Cells with LA EPEC
100
75
50
25
0
glucose
mannose
fructose
galactose

Figure 4.5. Growth curves for EPEC E2348/69 and JPN15 in carbohydrate-supplemented DMEM. At 1 h intervals, an aliquot of culture was removed and washed with PBS. The bacteria were then resuspended in PBS and the optical density of the suspension was measured at 600 nm. E2348/69 with glucose (—◆—), E2348/69 with galactose (—■—), and JPN15 with glucose (---○---).

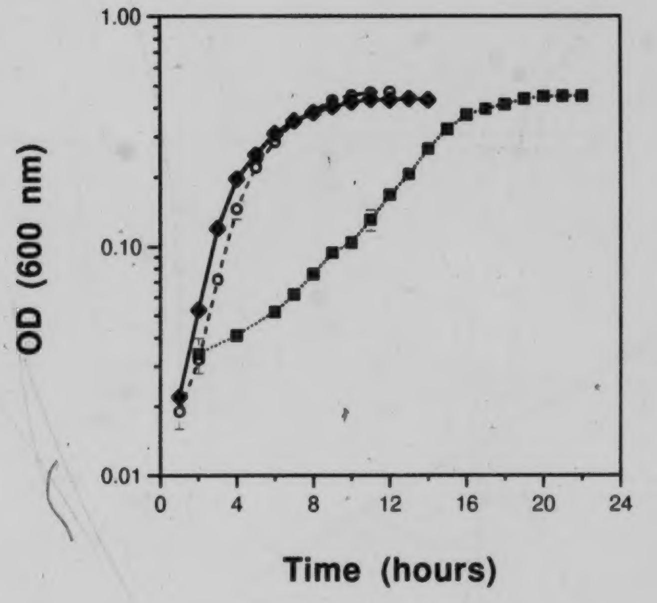

1.00
0.10
0.01
OD (600 nm)
0
4
8
12
16
20
24
Time (hours)

Figure 4.6. Quantitation of EPEC E2348/69 and JPN15 during log-phase growth in DMEM with glucose or galactose. At various stages of growth, an aliquot of culture was serially diluted in PBS and spread on tryptic soy agar plates to determine the number of CFU per ml of culture. JPN15 with glucose (▩), E2348/69 with glucose (■), and E2348/69 with galactose (☒).

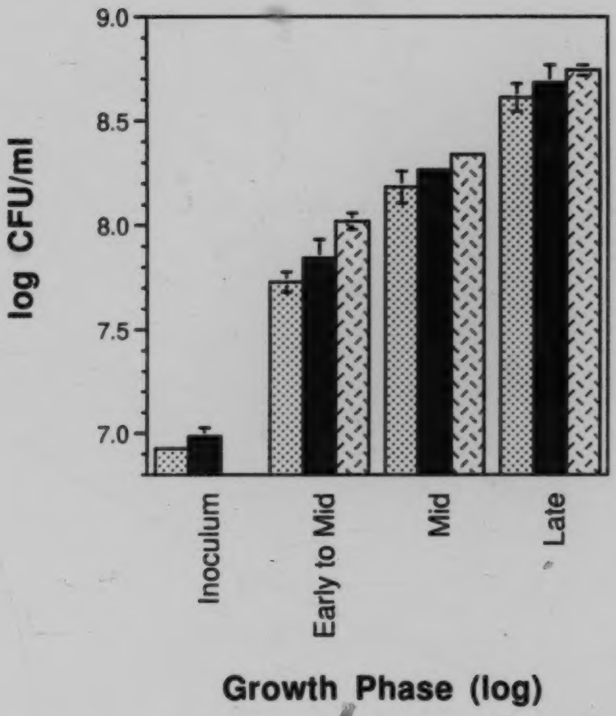

log CFU/ml
9.0
8.5
8.0
7.5
7.0
Inoculum
Early to Mid
Mid
Late
Growth Phase (log)

Figure 4.7. Growth curves for EPEC serotypes O111:H2 and O119:H6 in DMEM supplemented with glucose (—◆—) or galactose (—◇—). The optical densities of the various cultures were determined as described in the legend to Figure 4.5.

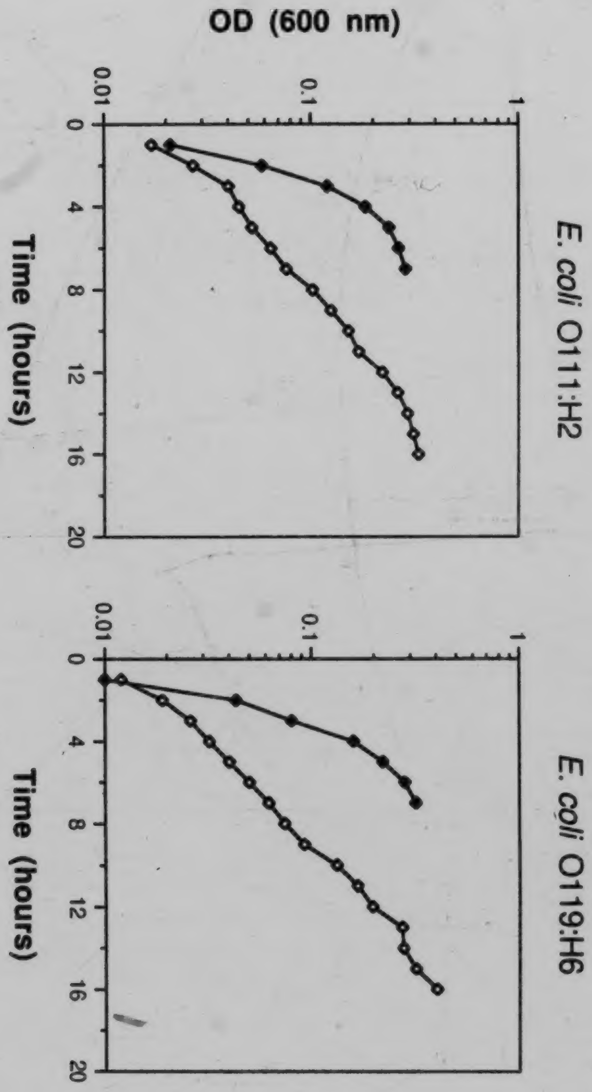
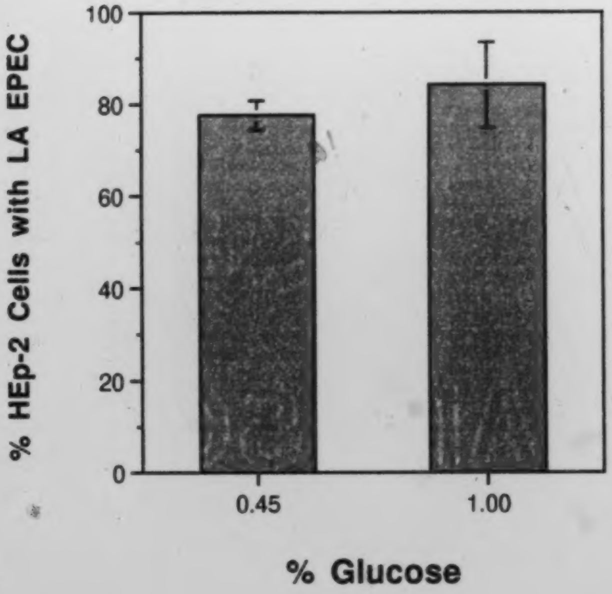

E. coli O111:H2
E. coli O119:H6
OD (600 nm)
0.01
0.1
1
0
4
8
12
16
20
Time (hours)
Time (hours)

Figure 4.8. Comparison of EPEC attachment when bacteria are grown in media with 0.45% or 1.0% glucose. Overnight TSB-grown organisms were cultured in DMEM with 0.45% or 1.0 % glucose for 2 h in a CO_2 incubator. The bacteria (900 μl) were then incubated with HEp-2 cells, in the presence of 1% mannose (100 μl), for an additional 30 min in a CO_2 incubator. After washing with PBS, the cells were fixed methanol and stained with Giemsa stain. The number of HEp-2 cells with LA EPEC was determined as described earlier.


% HEp-2 Cells with LA EPEC
100
80
60
40
20
0
0.45
1.00
% Glucose

CHAPTER 5

General Discussion

5. INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) adherence to the intestinal epithelium is associated with clinical symptoms of diarrhea. Since previous research suggested that EPEC bound to carbohydrate structures on eukaryotic cells, our studies focused on the identity, and role, of specific oligosaccharide sequences in attachment. In the following discussion, the results of these studies and their significance are summarized. As well, additional avenues of investigation are presented.

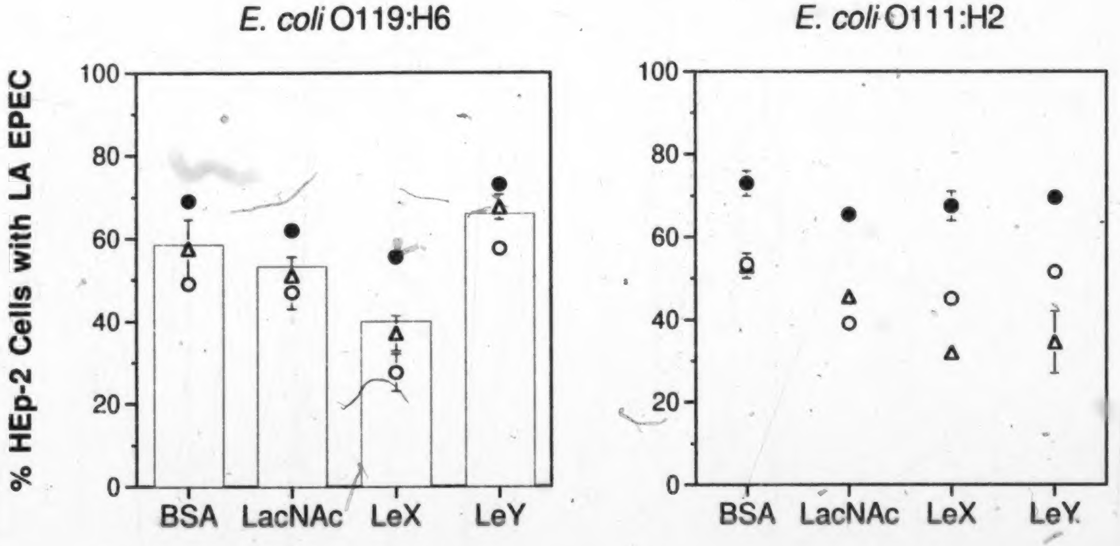
5.1. IDENTIFICATION OF CARBOHYDRATE STRUCTURES RECOGNIZED BY EPEC

Our results with Chinese hamster ovary (CHO) cell Lec mutants demonstrated that EPEC bound to lactosamine sequences on epithelial cells (Chapter 2). Although sialylated sequences were not necessary for recognition since EPEC bound in similar numbers to wild-type CHO and Lec2 cells, these sequences might be involved in eukaryotic cell signaling. Our binding results were consistent with previous reports which suggested that lactosyl sequences were recognized by EPEC (9, 23). In contrast to earlier reports, however, using this model system we also found that lactosamine sequences of N-linked glycoproteins, in particular, were receptors for EPEC. A role for these structures in initial, non-intimate attachment was suggested by the relatively short incubation period (1.5 h) required for a large number of EPEC to adhere to the CHO cells. (This incubation period was chosen since monolayer detachment occurred at later times). Intimin mediated attachment, in comparison, generally requires a co-incubation period greater than 3 h in order to observe significant levels of bacterial attachment (31).

To extend these results further, we performed binding inhibition experiments using synthetic, bovine serum albumin (BSA)-glycoconjugates to identify specific oligosaccharide sequences recognized by EPEC (Chapter 3). When EPEC E2348/69 (O127:H6) (33) was used in these experiments, we found that *N*-acetyllactosamine (LacNAc)-BSA was the most effective inhibitor of bacterial attachment. These results supported our earlier results obtained with the CHO and Lec cell lines. Lewis X (LeX)-BSA also inhibited bacterial attachment, but to a lesser extent.

Our earlier results with CHO and Lec cells indicated that EPEC of three different serotypes recognized lactosamine sequences of epithelial cells. Therefore, we performed binding inhibition experiments, in addition to those presented in Chapter 3, to determine whether a similar pattern of inhibition would be observed for these strains. In contrast to results obtained with EPEC E2348/69, we found that EPEC O119:H6 was inhibited to the greatest extent by LeX-BSA, which was followed by LacNAc-BSA (Figure 5.1). LacNAc-BSA was the most effective inhibitor of EPEC O111:H2 in two of three trials, while the reverse was observed in a third trial. While these results suggest that EPEC, as a group, may recognize more than one lactosyl structure, further experiments must be performed to eliminate other possible reasons for the observed differences. These include differences in the passage numbers of the organisms (45), or the geographical isolation of the organisms. Alternatively, conditions required to induce the expression of adhesins may be "sub-optimal and borderline" such that minor differences in assay conditions could result in differences in the binding specificities of the bacteria (27). Although our results using EPEC serotypes O127:H6 and O119:H6 were consistent between trials, it is conceivable that EPEC strains of different serotypes might differ in their responsiveness to

Figure 5.1. Glycoconjugate inhibition of EPEC serotypes O119:H6 and O111:H2. Experiments were performed essentially as described in the Materials and Methods of Chapter 3. After inoculating overnight, tryptic soy broth (TSB)-grown organisms into Dulbecco's modified Eagle medium (DMEM), EPEC O119:H6 or O111:H2 were cultured for 1 h 10 min or 30 min, respectively, at 37°C in a CO_2 incubator. Aliquots of the cultures were then incubated with the bovine BSA-glycoconjugates (final concentration of 0.6 mg/ml) for 30 min before both were transferred to tissue culture plate wells containing sub-confluent HEp-2 cell monolayers. The plates were incubated for an additional 30 min in a CO_2 incubator, after which time the cells were washed with phosphate-buffered saline (PBS), fixed with methanol, and stained with Giemsa stain. The number of HEp-2 cells with LA EPEC was determined as described earlier. Trial 1(●), Trial 2 (○), Trial 3 (△). Error bars represent the average of duplicate determinations for each sample. The overlaying bar graph indicates the average of the data obtained from the three independent trials.


E. coli O119:H6
E. coli O111:H2
% HEp-2 Cells with LA EPEC
100
80
60
40
20
0
BSA
LacNAc
LeX
LeY

an environmental cue(s), which is needed to induce the expression of a particular adhesin. Future experiments to elucidate the mechanism(s) for this diversity will be important because it will have implications (i.e. single versus multiple oligosaccharide sequences) for designing carbohydrate-based therapies for treating EPEC infection (27, 58).

For our studies with BSA-glycoconjugates, only a few lactosaminyl compounds were tested. This was done because an experimental protocol had to be developed before additional compounds could be tested. The specific carbohydrate structures used in these experiments were selected based on previous reports that N-linked glycoproteins of small intestinal epithelial cells of certain individuals consist primarily of lactosamine sequences in a β(1→4) linkage (17), and that EPEC preferentially recognized fucosylated lactosyl structures (9). However, since intestinal cells also express N-linked glycoproteins containing β(1→3)-linked lactosamine sequences (17), additional experiments should be performed using BSA-glycoconjugates presenting these oligosaccharides to determine whether these sequences are more effective inhibitors of EPEC binding. Ultimately, it would be preferable to identify a single carbohydrate structure that could inhibit the binding of a majority of EPEC isolates. Then, although the EPEC strains might display different sensitivities to inhibition by this structure, this sequence might reduce bacterial binding sufficiently to allow the host immune response to resolve the infection before clinical symptoms progress (27, 58).

5.2. ROLE OF LACTOSAMINE SEQUENCES IN ATTACHMENT

The results of our studies to identify oligosaccharide sequences which were receptors for EPEC also suggested that these structures have distinct roles in binding. Since EPEC did not bind to CHO cell Lec1 mutants, which lack lactosamine sequences on

N-linked glycoproteins, this indicated that EPEC physically bound to these structures when attaching to epithelial cells. By inference then, the decrease in EPEC binding observed in the subsequent binding inhibition experiments was likely, in part, due to the oligosaccharide sequences physically competing with host cell receptors for bacterial binding (2, 13).

On the other hand, results of experiments in which bacteria were co-incubated with the glycoconjugates alone, suggested that specific carbohydrate sequences might also reduce EPEC attachment by causing a decrease in the expression of EPEC bundle-forming pili (BFP) and intimin. Since, to our knowledge, a similar effect has not been described previously, these results provide another model for investigating systems within EPEC that regulate the virulence phenotype. Based on our current knowledge of EPEC pathogenesis, there are two regulatory mechanisms that may be involved in the down-regulation of BfpA and intimin observed in these experiments. The first mechanism involves the *per* (plasmid-encoded regulator) region of the EPEC adherence factor (EAF) plasmid (21, 25). The involvement of this region is suggested by results of Knutton *et al.* which demonstrated that EAF-plasmidless mutants did not down-regulate intimin levels in later stages of attachment, but regained this ability when reconstituted with the *per* region (30). It is interesting that in their studies, BFP expression was not down-regulated during the same period since earlier reports demonstrated that the *per* region positively regulates the expression of both of these proteins (21, 48). One possible explanation for these observations is that BfpA and intimin have different stabilities once the expression of these proteins is halted. However, our results that the expression of BfpA and intimin are affected to different extents by LacNAc-BSA and LeX-BSA, respectively, suggests that the expression of these proteins, in part, vary in their responsiveness to environmental (or host) signals.

A second possible mechanism by which the expression of BfpA and intimin might be reduced, though more speculative, is suggested by recent results of Farris *et al.* In their studies, they identified a tyrosine phosphorylated GTPase of EPEC, BipA (for bactericidal/permeability-increasing protein Δ (40)), which was involved in regulating several bacterial processes, including the formation of A/E lesions (14). It was speculated that BipA might regulate protein expression by interacting with bacterial ribosomes. These results are significant not only because they identify a specific protein involved in the regulation of virulence, but they also demonstrate general mechanisms which might be involved. These mechanisms are the GTPase activities of proteins (8), and the phosphorylation of bacterial proteins (56).

Overall, in order to determine if the *per* region, or BipA, are involved in down-regulating EPEC attachment in our experiments with the glycoconjugates, experiments should be repeated using mutants for these loci. An alternative approach would be to create transposon insertion mutants of wild-type EPEC which do not decrease their expression of BfpA or intimin when incubated with the glycoconjugate(s) (10, 36). The gene(s) interrupted by the mutation could be then be identified by sequencing the DNA flanking the insertion. In addition to these experiments, determining whether decreases in BfpA and intimin expression occurs at the transcriptional (26, 39) or translational level (25, 57), or if the stabilities of these proteins are altered (25, 57), will further our understanding of the regulation of these proteins.

With respect to EPEC pathogenesis, our experimental results are encouraging because they suggest a potential use for carbohydrate compounds to treat EPEC infection (27, 58). The advantage of using carbohydrate-based therapies, instead of treatments such as antibiotics, is that bacteria are unlikely to develop resistance to these compounds. From a bacterial perspective, once they bind to a carbohydrate structure, they "have found a host". Therefore, there is no selective pressure to modify their binding specificity. Although it is still possible that an organism with an altered adhesin could colonize the host, this occurrence is likely to be low (58).

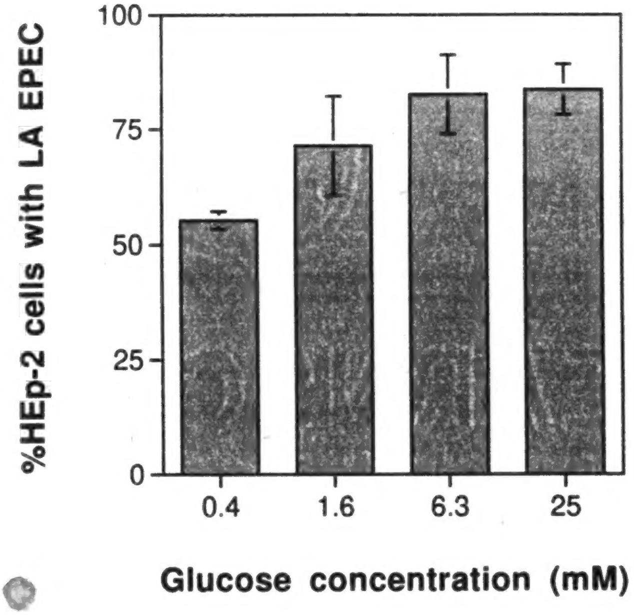
An important implication of our binding inhibition experiments is that a similar approach might be feasible for altering the expression of other EPEC virulence factors, or analogous virulence factors of other enteric pathogens. As mentioned in a previous section (Chapter 3), proteins exported by the type III secretory pathway of EPEC initiate signal transduction processes involved in the formation of A/E lesions (22, 24, 29). The ability to alter the expression of these proteins is suggested by results that their expression is also influenced by the *per* region (29), and by specific growth conditions which affect BFP and intimin levels (28). If this is demonstrated, similar strategies might also be effective in altering protein secretion by type III pathways of other enteric pathogens, including *Shigella*, *Salmonella*, *Yersinia* and EHEC (32). Other possible targets for regulation by glycoconjugates are the intimins expressed by the human enteric pathogens enterohemorrhagic *E. coli* (EHEC) (18, 49) and *Hafnia alvei* (1) which also cause A/E lesions. Since intimins display the greatest diversity at their C-terminus (6, 19, 26, 44, 54) which is associated with binding specificity, it remains to be determined whether LeX-oligosaccharide sequences will decrease the expression of intimin in these organisms.

5.3. METABOLIC REGULATION OF EPEC ATTACHMENT

Previous reports demonstrated that growth conditions affect the expression of EPEC virulence factors (20, 22, 28, 39, 42, 52). In our experiments, we found that carbon source also affected the expression of EPEC BfpA and intimin, and the ability of bacteria to bind (Chapter 4). One obvious consideration for the mechanism by which glucose or galactose exerted their effects relates to the different growth rates of EPEC in the different media (51). In general, variations in growth rate have been shown to affect the composition of bacterial outer membranes (53). This altered structure is presumed to be an adaptation by the bacteria to express structures which are appropriate for the environment they are in and which enable the bacteria to survive. That growth rate might be involved in regulating EPEC adherence is suggested by results of Puente *et al.* in which the expression of BfpA, the structural subunit of BFP, was maximal during log phase growth of the organisms (39). In their studies, Puente *et al.* attempted to determine whether the expression of BfpA was related to growth kinetics by culturing EPEC in media containing 0.1% or 0.45% glucose. Unfortunately, the bacteria had similar growth rates in both media so this question was not resolved. To address this issue then, additional binding experiments should be performed using media containing concentrations of glucose which alter the growth rate of EPEC (51). Since we found that glucose concentrations of 0.03% (1.6 mM) or less caused a reduction in EPEC binding (Figure 5.2) (growth rate was not examined since the purpose of these experiments was to examine EPEC binding under conditions similar to those in the small intestine), this concentration of glucose or lower would be appropriate for these experiments.

While the consideration of growth rates as a possible mechanism by which the expression of EPEC BfpA and intimin are regulated is logical, this approach is limited in that it presumes that a single regulatory process is involved. Since multiple regulatory pathways exist in bacteria (16), however, it is possible that more than one mechanism contributes to the overall effect of carbon source on BFP and intimin levels. Therefore, our laboratory is currently using an alternative approach to elucidate the regulatory pathway(s) responsible for the effects of carbon source on attachment. This approach involves creating transposon insertion mutants of wild-type EPEC which, when grown in medium

Figure 5.2. Effect of glucose concentration on EPEC adherence. After incubating overnight, TSB-grown organisms in DMEM with glucose for 2 h, 900 µl of bacterial culture and 100µl of 10% mannose (to prevent type I pili-mediated attachment) were added to wells containing subconfluent HEp-2 cell monolayers grown on glass coverslips. The cells were co-incubated for 30 min and then washed with PBS. After fixing with methanol, the cells were stained with Giemsa stain and the number of LA EPEC was determined.


%HEp-2 cells with LA EPEC
100
75
50
25
0
0.4
1.6
6.3
25
Glucose concentration (mM)

containing galactose, are able to attach to epithelial cells (10, 36). The gene(s) responsible for the altered phenotype will then be identified as described earlier.

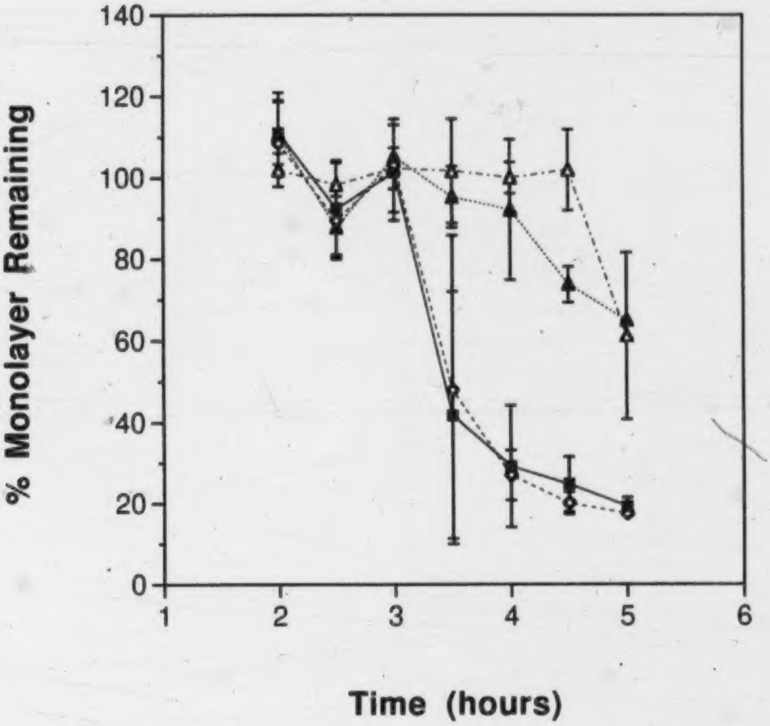
In general, it is thought that the expression of EPEC virulence factors is enhanced when bacteria are cultured under conditions similar to those found in the small intestine of humans (28, 39). Whether or not the availability of glucose could also be a signal by which EPEC sense a host environment is uncertain. A report by Olsen and Ingelfinger suggests that the levels of glucose in the small intestine of humans varies between 0.2 – 48 mM (15, 37). Based on these levels of glucose, we performed additional experiments to determine whether EPEC grown in medium containing similar levels of glucose could still attach to epithelial cells (Figure 5.2). Results revealed that EPEC grown in medium containing 0.4 mM (0.007%) glucose still attached to approximately 55% of the HEp-2 cells. Although levels of glucose less than those 0.4 mM were not used in these experiments, these results suggest that EPEC are able to attach to epithelial cells at relatively low concentrations of glucose. Therefore, even if glucose is not a direct signal by which EPEC recognize a host environment, it may provide sufficient nutrient to support bacterial growth and colonization of the small intestine. By extension, it is interesting to speculate that perhaps the higher levels of glucose in the small intestine, compared to the large intestine (38), could contribute to this site being preferentially colonized by EPEC (4, 43, 50).

5.4 DETACHMENT OF CHO CELL MONOLAYERS

In our experiments with CHO cells, we found that EPEC induced a rapid detachment of monolayers from the wells of tissue culture plates (Chapter 2). This effect did not appear to be related to toxicity since loss of the monolayers occurred between 2 - 4 h after the bacteria were added, but appeared to recover at later time intervals (4 - 6 h). In support of this conclusion, trypan blue staining of the monolayers at later time points revealed that the detached CHO cells were viable. It is of interest that these effects were only transient. This suggests that either the bacterial signal(s) occurs only for a limited period of time, or alternatively, eukaryotic cells have some ability to recover from effects associated with signaling.

Since CHO cell detachment was transient, we performed additional experiments to determine whether this effect was associated with eukaryotic signaling pathways which were triggered by EPEC binding. For these experiments, wild-type EPEC isolate E2348/69 (O127:H6) was used since mutants of this strain which are defective in various steps of attachment (and therefore signaling) were available (11, 24-26, 33, 34, 41). Briefly, *cfm* 27-3-2(1) has a mutation in the *sepA* gene of the type III secretory pathway. These bacteria attach to epithelial cells in comparable numbers to the wild-type strain, but do not secrete proteins required for inducing signals in eukaryotic cells. Consequently, these bacteria do not cause any cytoskeletal rearrangement. The strain *eaeA* 10-5-1(1) has a mutation in the gene encoding intimin. These bacteria also are not affected in their initial attachment to epithelial cells and are able to cause a diffuse accumulation of actin beneath adherent organisms. However, they are unable to focus the actin to form A/E lesions. JPN15 lacks the EAF plasmid required for initial attachment, but is still able to attach intimately to epithelial cells after prolonged periods of incubation. Results of these experiments (Figure 5.3) demonstrated that EPEC-induced signaling is required for CHO cell detachment since the mutant *cfm* 27-3-2(1) was unable to induce this effect. Significantly, however, the *eaeA* 10-5-1(1) mutant caused detachment similar to wild-type bacteria. This indicated that signaling processes shown to follow intimate attachment, including increases in intracellular calcium, activation of protein kinase C, and the phosphorylation of phospholipase $\gamma 1$, were not involved. Thus far, only the tyrosine

Figure 5.3. EPEC-induced signaling causes CHO cell monolayer detachment. Detachment assays were performed similar to those described in Materials and Methods of Chapter 2. Briefly, 5μl of overnight, TSB-grown organisms was inoculated into wells containing confluent monolayers of CHO cells. Mannose was included in this medium to prevent type I pili-mediated adherence. The plate was then incubated at 37°C in a CO_2 incubator, and at various time intervals, the monolayers were washed with PBS. After fixing the cells with methanol and staining with Giemsa stain, the cells were lysed with 2% SDS and the Abs_{620} of the solutions was recorded. The percentage of monolayer remaining was calculated as described in Chapter 2. Wild-type E2348/69 (—■—), JPN15 (----△----), *cfm* 27-3-2(1) (·······▲·······), and *eaeA* 10-5-1(1) (····◇····).


% Monolayer Remaining
0
20
40
60
80
100
120
140
1
2
3
4
5
6
Time (hours)

phosphorylation of Tir, the dephosphorylation of (as of yet) unidentified host cell proteins, and the generation of inositol phosphates have been shown to be early signaling (i.e. preceding intimate attachment) events. To identify if these, or other, signals are involved in mediating monolayer detachment, experiments should be repeated using specific chemical inhibitors of various pathways to identify those responsible for this effect (5, 7, 12, 41, 55).

One possible explanation for the loss of CHO cell monolayers is that this effect is related to the rearrangement of the cytoskeleton in these cells following EPEC attachment. This hypothesis is consistent with the studies of Nakamura *et al.* in which ammonium chloride was found to cause the detachment of several types of cells including fibroblast, epithelial and endothelial cell lines (35). In their experiments, as in ours, cell detachment was not a consequence of cell death. Investigations by this group into the reasons for cell detachment revealed that the loss of cells was associated with the reorganization of the cytoskeleton and phosphorylated proteins in these cells. In particular, increases in intracellular calcium and the dephosphorylation of several proteins were required for this effect. While it is uncertain whether EPEC-induced signaling could cause a generalized effect on the cytoskeleton which our hypothesis implies, perhaps the adherence of CHO cells to the tissue culture wells is sufficiently weak that minor disruptions in the cytoskeleton can cause cells to detach. Recently, Bain *et al.* proposed that the cytoskeletal rearrangement caused by EPEC during the formation of A/E lesions does not involve increases in intracellular calcium, but instead is mediated by proteins which are secreted by the type III system of bacteria (3). Their hypothesis was based on the observation that *Yersinia*, *Salmonella*, and *Shigella* species secrete proteins *via* similar pathways which induce similar effects in a calcium-independent manner. If CHO cell detachment is related to actin rearrangement, this model system would be useful to identify the bacterial factors involved in this effect.

Finally, using the CHO cell model system, it appeared that sialylated carbohydrate sequences on the surface of CHO cells were necessary for monolayer detachment. For these experiments, because of the different growth properties of the different Lec cell lines and wild-type CHO cells (46), the monolayers were incubated for different periods of time (i.e. 2 - 3 days) in a CO_2 incubator to obtain confluent monolayers. In retrospect, these differences could result in variations in the degree of confluency of the monolayers, which in turn could affect the adherence of the cells to the tissue culture wells. Since it would be difficult, if possible at all, to standardize the adherence properties of the cell lines, an alternative approach should be used to examine the role of sialylated sequences in detachment. This approach would be to enzymatically cleave sialic acid residues from the cell surface carbohydrates of wild-type CHO cells (47). Then if differences in detachment were observed with treated cells compared to untreated cells, the role of sialic acid in this effect could be further investigated.

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CHAPTER 6

General Conclusion

6. CONCLUSION

Overall, the results of our studies demonstrate that carbohydrates have several distinct roles in enteropathogenic *Escherichia coli* (EPEC) attachment. These results allow us to be optimistic that by a careful and thoughtful selection of carbohydrate-based therapeutic agents, we may be able to interfere with different stages of EPEC attachment and possibly affect the expression multiple virulence factors. Equally important however, are the questions that are raised by these results. Further investigations to answer these questions would contribute significantly to our understanding of EPEC pathogenesis.